Impact of sorbic acid and other mild preservation stresses on germination and outgrowth of *Bacillus cereus* spores

Clint C. J. van Melis

PhD Thesis Clint van Melis_corrected1.indd 1 4-12-2013 10:55:38

Thesis committee

Promotor
Prof. Dr T. Abee
Personal chair at the Laboratory of Food Microbiology
Wageningen University

Co-promotor
Dr M.N. Nierop Groot
Senior scientist, Food and Biobased Research
Wageningen University and Research Centre

Other members
Prof. Dr M. Kleerebezem, Wageningen University
Prof. Dr S. Brul, University of Amsterdam
Dr O.A. Økstad, University of Oslo, Norway
Dr M.H.J. Wells-Bennik, NIZO Food Research, Ede

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

PhD Thesis Clint van Melis_corrected1.indd 2 4-12-2013 10:55:38

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Clint C. J. van Melis

Thesis
submitted in fulfillment of the requirements
for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 20th of December 2013
at 1.30 p.m. in the Aula.

PhD Thesis Clint van Melis_corrected1.indd 3 4-12-2013 10:55:38

C.C.J. van Melis

Impact of sorbic acid and other mild preservation stresses on germination and outgrowth of *Bacillus cereus* spores
138 pages

PhD thesis, Wageningen University, Wageningen, NL (2013)
With references, with summaries in Dutch and English

ISBN 978-94-6173-775-5

PhD Thesis Clint van Melis_corrected1.indd 4 4-12-2013 10:55:38

Table of contents

PhD Thesis Clint van Melis_corrected1.indd 5 4-12-2013 10:55:38

Abstract

Weak organic acids such as sorbic acid, lactate, and acetic acid are widely used by the food industry as preservatives to control growth of micro-organisms. With the current trend towards milder processing of food products, opportunities arise for spore-forming spoilage and pathogenic microorganisms such as *Bacillus cereus*, that may survive the use of milder heating regimes. Dormant spores produced by *B. cereus* can survive processing conditions and their subsequent outgrowth increases the risk of premature spoilage and food safety issues. As a consequence, the use of additional preservation hurdles, such as acidification with weak organic acid additives to ensure the quality and safety of a product is important. Sorbic acid is widely used as an antimicrobial compound because of its strong inhibitory properties against bacteria and other spoilage organisms. Its effectivity has also been ascribed to its hydrophobic character, resulting in an additional mode of action not observed with other less lipophilic organic acids such as lactic acid and acetic acid.

In this project the impact of sorbic acid on spore germination and outgrowth was studied at transcriptome level and was linked to the distinct phenotypic responses observed for spores exposed to different levels of sorbic acid. The various stages of spore germination and outgrowth could be recognized by distinct gene expression profiles representing either the germination phase, transition state between germination and outgrowth or outgrowing cells, respectively. A subset of genes was specifically expressed in sorbic acid-exposed germinating spores and included functions related to cell envelope, (multi) drug transporters and amino acid metabolism. At high concentrations of sorbic acid (3mM of the undisssociated form, HSA), nutrient-induced germination of *B. cereus* ATCC 14579 spores was completely blocked. This blockage was shown to be reversible and could be bypassed by known non-nutrient triggers that activate spores in a receptor-independent way, pointing to a possible interference of HSA with the signaling event between germinant receptors and proposed SpoVA-channels, possibly by accumulation into the spore's inner membrane. Additional experiments with other inhibiting compounds, including organic acids and their structurally similar alcohol counterparts, showed that the lipophilic properties are an important determinant of its efficacy to block germination.

Building on current knowledge on the interaction of germination-relevant protein clusters, we discuss a hypothetical model on the mode of action of sorbic acid and other short-chain lipophilic compounds in germination inhibition of *B. cereus* spores. In addition to the interference or even blockage of germination, sorbic acid may increase outgrowth heterogeneity when applied at lower concentrations (0.25-1.5 mM) that still allow outgrowth. The first stages of outgrowth were shown to specifically occur heterogeneously when spores were exposed to multiple stresses simultaneously.

PhD Thesis Clint van Melis_corrected1.indd 6 4-12-2013 10:55:38

Heterogeneity effects were most pronounced for combined stress-effects where heat-treated spores were also exposed to low pH stress. Under these conditions, a large subpopulation of spores was delayed between initial germination and swelling and further outgrowth. For the food producing industry, it would be desirable to have reliable parameters to predict the behavior of surviving spores in a food product. Data presented in this thesis show that germination rate is not a good predictor for outgrowth heterogeneity when applied as a single indicator.

In conclusion, the work described in this thesis strive to obtain a better understanding on the impact that preservation stresses, including (sorbic) acid stress, have on the germination and outgrowth (heterogeneity) of *B. cereus* spores. The results obtained in this project may contribute to the rational design of new concepts for improved food preservation and safety.

PhD Thesis Clint van Melis_corrected1.indd 7 4-12-2013 10:55:39

PhD Thesis Clint van Melis_corrected1.indd 8 4-12-2013 10:55:39

Chapter 1

General introduction and thesis outline

PhD Thesis Clint van Melis_corrected1.indd 9 4-12-2013 10:55:39

1. Spore forming bacteria

Bacterial endospores are amongst the most resistant forms of living organisms. An endospore is a dormant, non-reproductive structure that is most commonly produced by bacteria from the Firmicute phylum. The endospore is a stripped-down, dormant form to which the bacterium can reduce itself in response to non-favorable conditions, most often nutrient limitation. The most commonly encountered endospore-forming bacteria are from the *Clostridium* and the *Bacillus* genus (Setlow and Johnson, 2007; Brown, 2000).

Bacterial spores are notorious for their formidable resistance against harsh conditions, including UV radiation, wet- and dry-heat, hydrogen-peroxide and certain chemicals (Setlow et al., 2006a; 2006b). Multiple layers of the spore are involved in this high level of resistance. In *Bacillus subtilis* the outermost layer of the spores is the coat, which has been shown to consist of at least four layers: a basement layer, a lamellar inner coat, a more coarsely layered outer coat, and a layer named the crust (McKenney et al., 2010; 2012), while in *Bacillus cereus* and *Bacillus anthracis* an additional layer has been identified, called the exosporium (Kailas et al., 2011; Steichen et al., 2003; 2005; Todd et al., 2003). The coat serves several roles to ensure dormant spores remain intact. For instance, it acts as a chemical filter by allowing access of small molecules including those that trigger germination such as amino acids and sugars, while being impermeable to large molecules that can damage the spore (Pedraza-Reyes et al., 2012). The next layer, the cortex, is located beneath the spore-coat and -outer membrane, and it mainly plays a role in coping with turgor pressure due to the high concentration of solutes in the core (Popham, 2002). The inner membrane acts as a strong permeability barrier against chemicals (Cortezzo and Setlow, 2004), while the spore core has multiple characteristics that contribute to its resistance. Resistance to wet heat, for example, is largely determined by the reduced water content of the spore core, while the level and types of mineral ions and specific spore proteins (small acid soluble proteins, SASP) protect the spore DNA from damage by radiation and chemicals (Pedraza-Reyes et al., 2012; Setlow et al., 2006a). The spore's resistance is largely influenced by conditions during the sporulation process, including environmental conditions (Melly et al., 2002; Garcia et al., 2010), nutrient- or chemical-based conditions (de Vries et al., 2005; Hornstra et al., 2006) or the pH of the sporulation medium (Wuytack and Michiels, 2001) that may all affect to different extends the parameters described above.

Due to the resistance of their endospores, members of the *Clostridia* and *Bacilli* have a large advantage over non-sporulating bacteria under adverse conditions, such as those typically encountered in food processing. Their high level of resistance allows the spores to survive thermal processing when in the pasteurization range, with some spore-formers surviving even more severe heating conditions, making efficient control of the survival and outgrowth of these spores of utmost importance to the food industry. Spore-formers relevant to the food industry-relevant are spoilage mesophilic and thermophilic *Bacillus* spp., and food-borne human pathogens like *Clostridium botulinum*, *Clostridium perfringens* and *B. cereus*. Of these spore-formers, the *Bacillus* genus has been studied most extensively, while the knowledge on resistance- and outgrowth-mechanisms in *Clostridium* spores is less extensive. *B. cereus* is frequently associated with food poisoning and food spoilage, and is therefore a representative model organism to study spore germination and outgrowth under stress conditions typically encountered in pro-

PhD Thesis Clint van Melis_corrected1.indd 10 4-12-2013 10:55:39

cessing of foods.

1.1 Bacillus cereus and the Bacillus cereus group

The *Bacillus cereus* group of organisms is a highly homogenous subdivision of the *Bacillus* genus, and includes *B. cereus*, *B. anthracis* and *B. thuringiensis*, *B. mycoides, B. pseudomycoides* and *B. weihenstephanensis* (Tourasse et al., 2006; Vilas-Bôas et al., 2007). Members of this subdivision have been studied extensively; both on the physiological and genetic level, due to their economic and medical importance. *B. cereus* is widespread in nature and its spores are commonly encountered in rice, cereals, spices and dairy products (Stenfors Arnesen et al., 2008). It is an opportunistic pathogen that is associated with two forms of human food poisoning (Stenfors Arnesen et al., 2008; Gould et al., 1995), causing diarrhea (Granum & Lund, 1997; Schoeni & Wong, 2005) or vomiting (Ehling-Schulz et al., 2004) and is also frequently encountered as a food spoilage organism (Andersson et al., 1995). A variety of adaptive stress responses, including the general stress response mediated via the sigma factor B-dependent regulon (van Schaik et al., 2005; 2004), allows *B. cereus* to adapt to diverse, often harsh, environments such as those encountered in the soil or the animal gut (Hong et al., 2009; Stenfors Arnesen et al., 2008).

The first two fully sequenced genomes of *B. cereus* were reported in 2003 and 2004 (Ivanova et al., 2003; Rasko et al., 2004). Nowadays, a total of 104 additional genome sequences have been described, of which 40 are fully completed, contributing to almost half of all the published *Bacillus* genomes. The increasing availability of genome-data on *Bacillus* spp. and improved platforms for DNA microarray technology has supported the application of transcriptome analysis significantly. This has led to an increasing number of studies on whole genome expression profiling of germinating *B. subtilis* spores and vegetative cells (Keijser et al., 2007; Ter Beek et al., 2008) and *B. cereus* cells (Mols et al., 2010a; 2010b; den Besten et al., 2009, van Melis et al, 2011 (chapter 2)), both in the absence and presence of mild preservation stresses.

1.2 Sporulation

During nutrient depletion, sporulation is induced and the bacterial cell undergoes a number of morphological changes, triggered by the expression of a cascade of sporulation specific genes, tightly controlled by the master transcription factor Spo0A and its level of phosphorylation via histidine kinases (including KinA, KinB and KinC) (Fujita and Losick, 2005; Mirouze et al., 2011; Pigot and Hilbert, 2004; Sonenshein, 2000). Phosphorylated Spo0A controls a large set of over 100 genes including those involved in asymmetric cell division and in expression of sigma factors specifically involved in sporulation (Molle et al., 2003). Following the trigger to initate sporulation, the bacterial cell undergoes asymmetrical division by the formation, and later curving and separation of an asymmetric septum, resulting in engulfment of the forespore by the mother cell (Higgins and Dworkin, 2012). After septum formation, the first compartment-specific transcription factor, σF is activated. The activation of σF, and the ~50 genes it controls, is a critical checkpoint, since it regulates the activation of the other sporulation specific sigma-factors that, in turn, regulate the next steps in the sporulation process (Wang et al., 2006; Piggot and Hilbert, 2004). The asymmetrical septum formation is followed by a period of transient chromosomal asymmetry and differential compartment-specific gene expression. The chromosomal asymmetry is followed by activation of σE, which controls ~260 specific

PhD Thesis Clint van Melis_corrected1.indd 11 4-12-2013 10:55:39

genes including SpoIIR which plays an essential role in cell to cell communication between the mothercell and the forespore (Eichenberger et al., 2004). Active σE leads to σG activation in the forespore, controlled by σF, which in turn leads to activation of σK in the mothercell. This inter-compartment communication and specific activation of sigma-factors is necessary for correct engulfment of the forespore, and further gene expression required for construction of the spore-specific components, such as the cortex and the spore-coat (Robleto et al., 2012). During maturation of the spores, they reach a state of metabolic dormancy, allowing them to survive in non-favorable environments for long periods of time while still being able to continually monitor the nutritional state of their surroundings and, when circumstances turn to favorable, can rapidly resume metabolic activity by a process called germination.

1.3 Spore germination

The process by which dormant spores wake up and re-initiate growth is called germination. Germination is commonly triggered in the presence of appropriate amounts of (mixtures of) nutrients, including simple amino acids, sugars, and/or nucleosides and is mediated by specific germination receptors (GRs; Moir, 2006; Setlow, 2003). Fig.1 shows a schematic representation of the different layers of the *B. cereus* spore, and the elements that comprise the typical germinant receptor in *Bacillus* spp. The genome of the type strain of *B. cereus* (ATCC 14579) encodes seven putative germinant receptors (Hornstra et al., 2006), of which five are highly conserved within members of the *B. cereus* group (Abee et al., 2011; van der Voort et al., 2010). These germinant receptors encompass three subunits (A, B and C, see Fig. 1), of which the first two are (partial) trans-membrane proteins and the latter is a lipoprotein that is most likely attached to the spore inner-membrane, facing the cortex side (Korza and Setlow, 2013; Moir et al., 2002; Ross and Abel-Santos, 2010).

Table 1 shows the germinant receptors that have been identified in *B. cereus* and *B. subtilis*

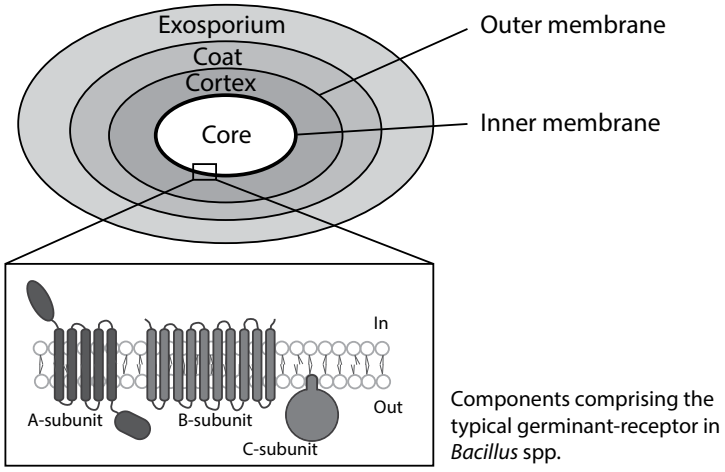


Fig. 1: Overview of the various layers that comprise the *B. cereus* spore. The thickness of the various layers can significantly vary between species. Furthermore, not all *Bacillus* species equip their spores with an exosporium. The magnified box shows the schematic structure of the three elements in the spore's inner membrane that form the typical germination receptor in *Bacillus* spp. (Adapted from Hornstra PhD thesis)

PhD Thesis Clint van Melis_corrected1.indd 12 4-12-2013 10:55:39

spores, and their currently known germinant triggers. In *B. cereus* ATCC 14579 the GRs GerR and GerG mediate the response to either L-alanine, L-cysteine and L-threonine in case of GerR and to L-glutamine in the case of GerG, and three of the putative GRs have been shown to be involved in purine riboside-induced germination by interaction with inosine and adenosine (GerR, GerQ and GerI), or by interaction with a mixture of amino acid and purine riboside, in this case L-phenyl-alanine + inosine (GerI) (Hornstra et al., 2005; 2006). The spore requires tight control mechanisms to prevent premature germination. Once such system was identified in the *B. anthracis* exosporium, where an alanine racemase converts L-alanine to D-alanine in order to prevent premature germination of the spore during maturation (Chesnokova et al., 2009).

The primary functions of germinant receptors are sensing the presence of nutrients and, upon nutrient binding, transduce a signal to trigger further processes in the germination pathway. Despite considerable research efforts, the exact mode of action of signal transduction is currently unknown and remains to be elucidated. However, a role for SpoVA in interaction with germinant receptors (Vepachedu and Setlow, 2007a; 2007b), Ca-DPA accumulation in the spore core during sporulation and in the release of Ca-DPA (via putative SpoVA channel)

Table 1: Currently known germinant-receptors in *B. subtilis* and *B. cereus* ATCC 14579 and the germinants that activate them.

Organism	Receptor	Amino acid germinants[a]	Purine germinants[a]
B. subtilis	GerA	L-alanine	
	GerB	AGFK[b]	
	GerK	AGFK[b]	
B. cereus ATCC 14579	GerR*	L-alanine L-cysteine L-threonine	Inosine/adenosine
	GerG	L-glutamine	
	GerQ		Inosine/adenosine
	GerI*	L-phe + inosine[c]	Inosine/adenosine
	GerK*		
	GerL*		
	GerS*		

[a] Concentrations of 1mM
[b] Mixture of asparagine, glucose, fructose and K^+
[c] Only with sub-germinal concentrations of inosine
* Highly conserved within the *B. cereus* group

PhD Thesis Clint van Melis_corrected1.indd 13 4-12-2013 10:55:40

during initiation of germination (Li et al., 2012; Wang et al., 2011a) has been reported.

A model for the nutrient-induced germination process is shown in Figure 2, and can be divided into two distinct stages: stage I is comprised of the early germination events, including receptor-mediated changes to permeability of the spore inner membrane, resulting in the efflux of ions and other small molecules from the spore core and the influx of water. Stage II comprises the later steps in germination, where degradation of the coat and cortex allow further hydration and swelling of the spore core. The first step in the initiation of germination is the interaction of the germinants with a (combination of multiple) germination receptor(s), located in the spore inner membrane (Paidhungat and Setlow, 2001a; Hudson et al., 2001). The recognition and binding of the germinant to the germination receptor irreversibly commits the spore to germination. Once a spore is committed to germinate, the process is irreversible and germination continues even when the original nutrients are removed. Commitment is often triggered within a short time span, commonly in the range of minutes or even seconds (Stewart et al., 1981; Wang et al., 2011a; Yi and Setlow, 2010).

Commitment is followed by the release of monovalent cations (H^+, Na^+ and K^+) and the large supply of pyridine-2, 6-dicarboxylic acid (dipicolinic acid, or DPA) chelated with divalent cations, such as Ca^{2+} from the spore core, mediated through putative SpoVA channels (Vepachedu and Setlow, 2005; 2007). This coincides with the release of free amino acids, including arginine, glutamic acid and other small molecules that reside in the dormant spore (Setlow et al., 2008b). As a result, the core pH increases (Setlow and Setlow, 1980; Swerdlow et al., 1981; Van Beilen et al., 2013) and, in combination with the rehydration of the core, allows for conditions that support re-initiation of metabolism (Setlow, 2003). The release of DPA and parallel uptake of water reduces the heat resistance of these partially germinated spores, but is not yet sufficient to fully allow enzymatic activity (Sunde et al., 2009; Setlow et al., 2001). At this point, stage I of the germination pathway is considered to be completed. The major event in stage II of the germination process is the degradation of the cortex peptidoglycan, initiated by two germination-specific peptidoglycan hydrolases, SleB and CwIJ (Setlow, 2003). This depolymerization of the cortical peptidoglycan by cortex-lytic enzymes removes a physical constraint on the spore core, allowing for further water uptake, expansion of the core and subsequent expansion of the inner membrane and germ wall, in turn permitting enzyme action and ending dormancy (Christie, 2012).

In addition to the two physiological pathways of germination, spore germination can also be triggered in the presence of chemical compounds or mechanical triggers that can bypass one or more of the individual components of the nutrient- or peptidoglycan-induced germination pathways (Fig. 2, left panel, shown in grey). Exposure to moderate hydrostatic pressure was shown to trigger germination via two distinct mechanisms depending on the amount of pressure applied. A pressure of 100 – 200 MPa stimulated germination of *B. subtilis* and *B. cereus* spores by activating the germinant receptors in the absence of nutrients (Wytack et al., 2000; Paidhungat et al., 2002), with different receptors displaying varying levels of sensitivity (Black et al., 2007) and activation being non-selective for specific germinant receptors (Black et al., 2005; Hornstra et al., 2005; Wei et al., 2009). A pressure of 500 MPa or higher, on the other

PhD Thesis Clint van Melis_corrected1.indd 14 4-12-2013 10:55:40

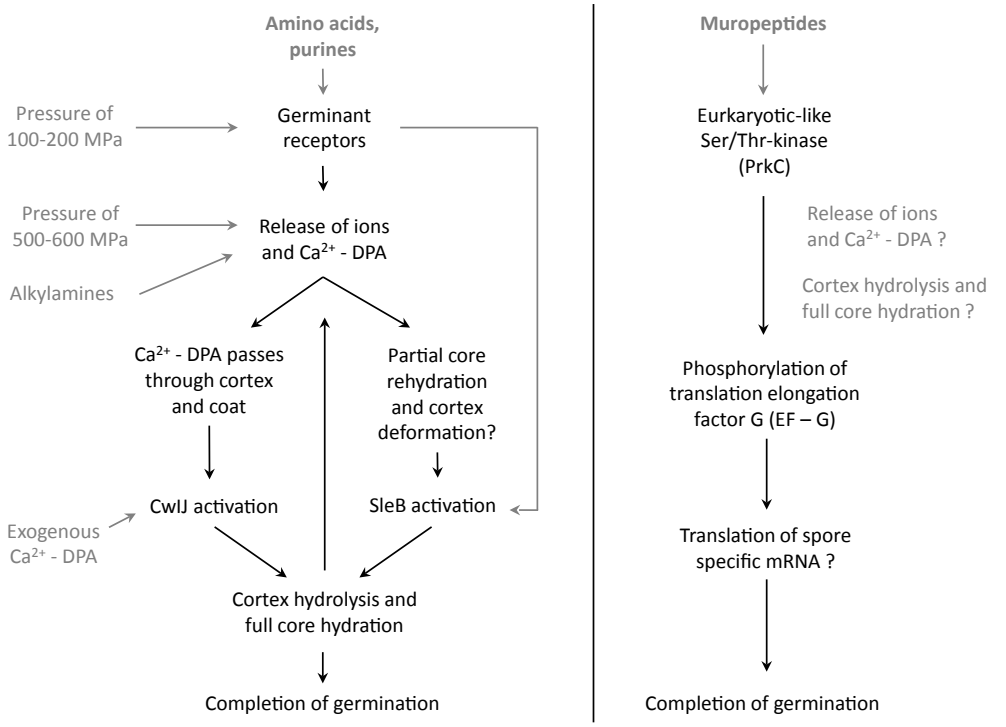


Fig. 2: Model of spore germination pathways in *Bacillus* spp. Both the nutrient-induced (left) and muropeptide-induced (right) pathways are shown. In the nutrient-induced pathway, amino acids and/or purines activate the germinant receptors, which causes the release of ions, including Ca^{2+} - DPA from the spore core which, in turn, triggers CwIJ action. SleB may be triggered by germinant receptor activation upon nutrient binding, and/or by the effects of core rehydration. Both enzymes then catalyze cortex hydrolysis, which allows for full rehydration of the core and completion of stage II of the germination process. Non-nutrient triggers (shown in grey) can activate germination on different levels of the germination machinery. The muropeptide-induced germination pathway is mediated by specific eukaryotic-like serine/threonine kinases (PrkC), located in the spore's inner membrane. Binding to PrkC causes the phosphorylation of translation elongation factor G (EF-G), which may or may not translate spore specific mRNA, ultimately resulting in the completion of germination. It is currently not known if phosphorylation of EF-G is the only path towards completion of germination, or if binding of muropeptides to PrkC also results in other signaling cascades. In addition, it is also not known where in this pathway the release of ions and Ca-DPA and full core rehydration occurs. (adapted from Setlow, 2003)

hand, was shown to completely bypass the germinant receptors but still trigger germination by stimulating the release of DPA, coincidentally activating cortex hydrolysis. Interestingly, low pressure of ~100 – 150 MPa did not induce germination in *C. perfringens* SM101 spores (Akhtar et al., 2009), and due to the differences in activation of the cortex lytic machinery in *C. perfringens* it can be expected that moderately high levels of pressure (500-600 MPa) also does not trigger germination (Sarker et al., 2013). Alternatively, germination can be triggered by exposure to cationic surfactants such as dodecylamine (Vepachedu and Setlow, 2007b; Setlow, 2003), that presumably upon insertion in the membrane cause (indirect) opening of the DPA-channels. A final non-nutrient trigger for germination is to by-pass the DPA-channels by exposure to high levels of exogenous Ca-DPA, leading to activation of cortex lytic enzymes and subsequent cortex degradation (Paidhungat et al., 2001b).

PhD Thesis Clint van Melis_corrected1.indd 15 4-12-2013 10:55:40

In addition to these germination triggers through the traditional germination pathway, recent studies have discovered a second germination pathway, mediated via eukaryotic-like serine/threonine kinase (PrkC) that is widely conserved in *Bacilli* and *Clostridia*, and which is activated upon binding specific peptidoglycan fragments, (Fig. 2; Setlow, 2008; Shah et al., 2008; Lee et al., 2010). Binding of the appropriate muropeptide fragments causes the PrkC-kinase to phosphorylate the ribosomal GRPase, elongation factor G, or EF-G (Squeglia et al., 2011), leading eventually to germination. The exact molecular details of how the completion of germination is reached after phosphorylation of the elongation factor remain to be elucidated.

2. *Bacillus cereus*, a spore-former in the food industry

In the food industry's search for more effective and less intense preservation as a basis for product innovation, spores including those of *B. cereus*, can cause major problems (Markland et al., 2013; Setlow and Johnson, 2007; Brown, 2000). The use of milder heating regimes allows spores to survive and in some cases even induces germination (Løvdal et al., 2011; Zhang et al., 2010; 2009). In the ready-to-eat food sector, *B. cereus* and other endospores formers are a cause of concern. These products are known as cooked-chilled foods or as refrigerated processed foods of extended durability (REPFEDs). *B. cereus* and other endospore formers are introduced in such products via vegetables, fruits, or herbs and spices (Heyndrickx, 2011). These products are typically processed with mild heat processing and depend on storage at refrigerator temperatures for preservation, allowing bacterial spores to survive and, in case of temperature abuse, to grow out causing premature spoilage of products (Carlin et al., 2000; Wijnands et al., 2006).

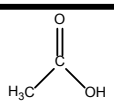
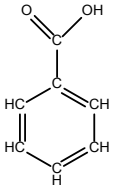
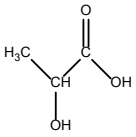
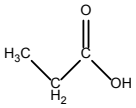
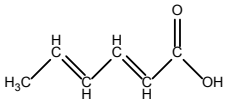
A second challenge for the food industry is the capacity of *B. cereus* to form biofilms. Biofilm formation may be initiated from the relatively hydrophobic spores that easily attach to pipelines and surfaces in the processing plant and are difficult to remove (Karunakaran and Biggs, 2011; Lequette et al., 2011; Ryu and Beuchat, 2005; Wijman et al., 2007). The (unprocessed) products typically contain sufficient levels of germinants, triggering germination and subsequent vegetative growth (Shaheen et al., 2010; Lindsay et al., 2006). Vegetative cells can then form multi-cellular biofilm complexes, wherein new spores can be formed and released in the liquid flow, thereby recontaminating processed products (Shaheen et al., 2010; Wijman et al., 2007; Andersson et al., 1995).

2.1. Preservation through multiple 'hurdles' to control spore formers

Consumer demands for milder processed foods which are appreciated as being more natural and healthy, and the drive to optimize cost-effectiveness of processing combined with sustainable processing, have led the food industry to focus on less intense heating regimes during processing. With the use of less intensive heating, additional preservation-methods, or 'hurdles' (Leistner, 2000; Leistner and Gorris, 1995), such as low concentrations of salt or acidification with (natural) additives become important. The aim of combined concepts is to inactivate or prevent outgrowth of the sub-lethally damaged spores with the additional stress of lowered pH, instead of allowing full recovery which would be possible under optimal conditions. From a safety point of view, the pH of a product should be as low as possible to prevent vegetative growth, but this will be less desirable from the consumer's perspective, since the pH of a food product has a strong impact on its organoleptic properties. It is therefore important that a

PhD Thesis Clint van Melis_corrected1.indd 16 4-12-2013 10:55:40

Table 2: Organic acids commonly used in the food industry.

Organic acid	Structure	pK_a	$logP_{o/w}$
Acetic acid		4.75	-0.17
Benzoic acid		4.20	1.87
Lactic acid		3.86	-0.47
Propionic acid		4.87	0.33
Sorbic acid		4.76	1.33

suitable balance between quality of a food product and optimal control of bacteria is found, since several spoilage and pathogenic organisms, including *B. cereus*, are able to adapt to mild pH stress (Browne and Dowds, 2002; Gould, 2000) In fact, sporeformers like *Alicyclobacillus acidoterrestris* are able to survive up to pH values as low as 2 (Chang and Kang, 2004), implying that low pH values alone may not be sufficient to prevent spoilage of a food product.

A common approach to control spores at acceptable pH levels, is to combine low pH with the presence of weak organic acid additives. Table 2 shows the most common weak organic acid additives used in foods including sorbic acid, benzoic acid, propionic acid, lactic acid and acetic acid (Theron and Lues, 2007; Beales, 2004). In general, these additives are more effective at low, rather than at high pH, with the rationale being that they work via the principle of the 'weak acid theory'. In this classical concept, weak organic acids exist in equilibrium between a dissociated and undissociated form, which is dictated by the pKa value of the organic acid and the pH of the environment. Consequently, in more acidic environments the equilibrium is shifted to the undissociated form (Fig. 3). Only uncharged molecules, such as the protonated form of the acid molecule, can diffuse freely across the membrane lipid bilayer. The protonated molecule will dissociate inside the cell, where it encounters an internal pH that is typically near neutral pH and higher than the pKa value of the additive, which usually ranges between 4.2 and 4.9 (Brul and Coote, 1999; Lambert and Stratford, 1999; Bogaert and Naidu, 2000; Marshall et al., 2000; Russell and Gould, 2003). Inside the cell a new equilibrium is formed between the dissociated and undissociated forms of the additive, and since entry

PhD Thesis Clint van Melis_corrected1.indd 17 4-12-2013 10:55:40

of the undissociated form into the cell is driven by diffusion, the process will continue until the concentrations inside and outside the cell are equal. An example of the impact of pH on the shift in equilibrium of the dissociated and undissociated forms of sorbic acid is illustrated in Fig. 3 (right panel). The protons that are released into the cytosol as a result of the dissociation of the protonated acid molecules can, depending on the buffering capacity of the cell, lower the internal pH and the proton gradient and in this way affect relevant biochemical processes. The resulting acidification of the cytosol and accumulation of anions is thought to be the main mode of action of weak organic acids (Beales, 2004; Booth and Stratford, 2003; Russell and Gould, 2003). In contrast, early work by Eklund (1983) showed that the dissociated form of an acid, in this case sorbic acid, in itself can also have an inhibiting effect. In the case of lipophilic acids, there appears to be a correlation between their lipophilic character and their efficiency in inhibition (Yasudo-Yasaki et al., 1978), possibly by interfering with membrane integrity and its functioning (Chu et al., 2009; Stratford and Eklund, 2003; Brul et al., 2002; Stratford and Anslow, 1998). This suggests that the effectivity of weak organic acids can be based on multiple factors, with the exact mechanisms of inhibition for these acids on spores remaining to be elucidated.

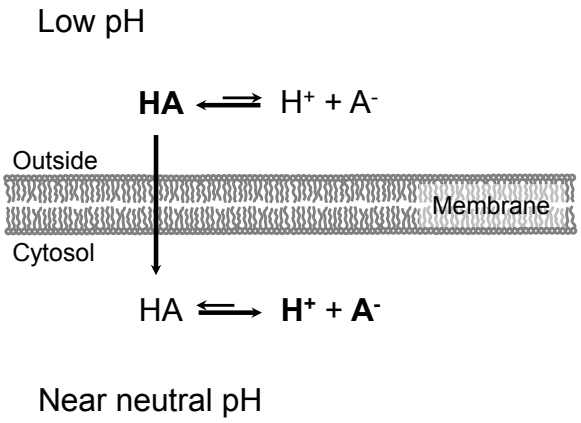
2.2. Implications of minimal processing

An example of products where the food industry is making strong efforts to reduce preservation intensity, are the ready to eat (RTE) foods. For many years, salt has been used as a preservation hurdle, but in recent years the focus of the food industry has been on reducing salt levels in order to produce more healthy foods. The reduction of salt levels in preservation concepts can have a relevant impact on the adaptation and survival capacity of stress-exposed cells and outgrowing spores. In fact, mild, non-lethal, stress has been shown to provide cross-protective effects towards other stresses (den Besten et al, 2010a; 2010c; 2013; Browne and Dowds, 2001; 2002), increasing the chance of survival and even growth of germinated spores. This is of practical relevance for minimally processed foods, because activation of stress response mechanisms in response to a mild stress can enhance resistance against other hurdles, as has been observed for a wide range of food-related microorganisms (Cebrián et al., 2010; Shen et al., 2011; van Bokhorst-van de Veen et al, 2011l; den Besten et al., 2013; Bergholz et al., 2012).

B. cereus displays adaptive responses controlled by alternative sigma factors that involve the expression of specific groups of genes, or regulons, involved in a wide range of functions, including heat-shock response, sporulation and general stress response (Marles-Wright and Lewis, 2010; Hecker et al., 2007; van Schaik and Abee, 2005). Rapid expression of these regulons is triggered by a wide range of physiological signals including temperature, pH, ethanol or osmotic stress and will often protect against multiple of these physiological insults (Abee and Wouters, 1999).

Exposure to non-lethal levels of salt during processing, for example, may induce the accumulation of so-called 'compatible solutes', such as betaine or carnitine, that are readily available in many food products (Smith, 1996). These compounds typically allow for growth at reduced water activity, but an additional effect of these compounds is that they can also cross-protect against cold-shock, as has been observed in Listeria monocytogenes (Ko et al., 1994; Angelidis

PhD Thesis Clint van Melis_corrected1.indd 18 4-12-2013 10:55:40



$$pH = pKa - Log\ \frac{[HA]}{[A^-]}$$

Sorbic acid, pK_a 4.76

pH7, 10mM total conc.
- 0.05 mM HA
- 9.95 mM A^-

pH5.5, 10mM total conc.
- 1.54 mM HA
- 8.46 mM A^-

Fig. 3: Overview of the mode of action of organic acids, as assumed in the 'weak acid theory'. The left panel shows the shift in equilibrium when the undissociated form of the acid (HA) passes through the inner membrane into the cytosol, where it uncouples into protons (H^+) and anions (A^-) due to the higher internal pH. The equation in the right panel shows the equilibrium between the dissociated and undissociated forms of an organic acid. This equilibrium is dependent on both the pH of the environment and the dissociation constant (pKa) of the organic acid. An example is given of the shift in equilibrium for 10mM of sorbic acid when the environmental pH is shifted from 7 to 5.5.

and Smith, 2003). Exposure to non-lethal salt concentrations during processing, may therefore prepare spoilage organisms and food-borne pathogens for growth at refrigerator temperatures. This principle of pre-adaptation due to mild, non-lethal stresses has also been shown to apply to other forms of stress. For instance, Den Besten et al. (2010a; 2006) showed that culturing cells of several *B. cereus* group members in the presence of sub-lethal levels of salt enhanced the thermotolerance of these cells. In addition, mild oxidative stress increased the robustness of B. weihenstephanensis KBAB4 cells against severe heat- or acid-stress (Den Besten et al., 2013) and mild heat stress enhanced the robustness of *B. cereus* ATCC 14579 cells against lethal heat- and acid-stress (Den Besten et al., 2010c).

2.3 Germination- and outgrowth-heterogeneity

Germination of a dormant spore population often occurs as a heterogeneous process (Eijlander et al., 2011; Stringer et al., 2011; Barker et al., 2005). Heterogeneity in germination and outgrowth arises when not all spores germinate and/or initiate outgrowth at the same time. One factor that causes heterogeneity is that not every spore within a dormant population displays the same level of sensitivity to specific germinants, resulting in a percentage of spores germinating rapidly, while another part, the so-called superdormant spores, remains dormant when exposed to germinants (Ghosh and Setlow, 2009, 2010). The cause for this heterogeneity is currently not fully understood. The number of germinant receptors in the dormant spore and the levels of cations available during sporulation have been hypothesized to play a role (Ghosh et al., 2012), but recent evidence showed that this is not the primary cause of germination heterogeneity (Zhang et al., 2013). Furthermore, in addition to germination-heterogeneity, there can also be considerable variability in lag periods between the different phases of the outgrowth of germinated spores (Yi and Setlow, 2010; Stringer et al., 2005). Heterogeneity, both in the germination- and outgrowth-phases, results in a population-wide distribution of outgrowth

PhD Thesis Clint van Melis_corrected1.indd 19 4-12-2013 10:55:41

behavior that differs from a normal distribution. A number of spores may grow out faster than the majority of the population, for example, representing those spores that may give rise to premature spoilage of products, or, alternatively, superdormant spores that survive processing and grow out at a later time-point, while the majority of the spore population was killed due to fast germination-behavior.

Germination- and outgrowth-heterogeneity complicates the prediction of outgrowth behavior considerably, especially when growth initiates from low numbers of spores, where fast outgrowing spores cause unexpected spoilage and/or safety issues. A large number of factors can play a role in outgrowth heterogeneity, including both historical stresses during sporulation and contemporary stresses in the spore's environment. For example, the temperature at the time of sporulation plays an important role in the level of heat resistance of the dormant spore (Melly et al., 2002; Condon et al., 1992), which in turn determines the survival rates of a spore population under processing conditions. Additionally, resistance to environmental- or processing-stresses is influenced by osmotic conditions (Nguyen Thi Minh et al., 2008; Webb et al., 2007) and the levels of cations available during sporulation (Ghosh et al., 2011).

Stresses evoked during food processing can also have a strong impact on the performance of outgrowing spore populations. Mild to severe heat treatment, for example, has been shown to generate subpopulations of sub-lethally damaged *B. cereus* spores from dormant spore populations (Cronin and Wilkinson, 2008). Other examples of heat-induced heterogeneity have been described for populations of *B. subtilis* (Smelt et al., 2008) and *C. botulinum* (Stringer et al., 2011), with a positive correlation between degree of stress and the amount of heterogeneity. Similar observations have been made for *C. botulinum* spores in the presence of salt (Webb et al., 2007) and vegetative cells of *B. cereus* (den Besten et al., 2007, 2010b). Less intensive, non-lethal, heating regimes or the reduction of salt concentrations, and possibly other types of stress as well, may thus increase heterogeneity which further complicates the prediction of outgrowth behavior. This underlines the importance of understanding the impact that these milder, non-lethal approaches to food preservation have on spore-germination and outgrowth behavior.

2.3.1 Toolbox for studying heterogeneity

The above mentioned non-uniform, heterogeneous, response of a spore population is masked in conventional microbial culturing methods, since these techniques rely on the averages of data collected from a large number of cells. Indirect and direct methods have been developed to study heterogeneity at a population or single cells/spores level. Conventional techniques, such as plate counting, have been combined with flow cytometry to study germination heterogeneity under selected stress conditions, including heat treatment (Cronin and Wilkinson, 2010, 2008, 2007; Stecchini et al., 2009; Reis et al., 2005). Distribution times of single cells or spores can be monitored by using optical density (OD) measurements in high throughput formats. One cell or spore per well can be either assumed based on diluting grown cultures or spore crops such that only one cell or spore is expected to be present per aliquot (Stringer et al., 2005) or by using flow cytometry, coupled to cell-sorting devices (Smelt et al., 2008). Changes in optical density are continuously monitored and are used to estimate lag times of the individual cells/

PhD Thesis Clint van Melis_corrected1.indd 20 4-12-2013 10:55:41

spores, typically by using time-to detection approaches or OD-based growth curves. The time of first cell division is estimated by extrapolation, and the variability between the individual outgrowth-curves is used to determine the variability in outgrowth-behavior of single spores. This indirect approach, where the initial outgrowth phase cannot be observed directly, depends on several assumptions, the most important ones being that all cells continuously grow at the same speed and that each well contains only one cell/spore. These approaches have provided relevant insights in outgrowth heterogeneity of several *Bacilli* (Smelt et al., 2008; Brousolle et al., 2008) and *Clostridia* (Stringer et al., 2005, 2011; Webb et al., 2007) spores.

In order to study heterogeneity in more detail, direct methods that enable measuring the initial stages of outgrowth, are required. For this purpose, microscopy has proven valuable since it allows direct visualization of heterogeneity in the lag time to germinate. Dormant spores are refractile when observed with phase-contrast microscopy, and lose this refractability when they take up water as a result of germination. This phenomenon is used in laboratories worldwide as a non-invasive method to study germination (Leuschner and Lillford, 1999; Chea et al., 2000; Kong et al., 2011; 2010; Adam et al., 2011). More recently, new methods have been designed that make it possible to study germination kinetics of individual spores. These methods include laser tweezers Raman microspectroscopy (Chen et al., 2006; Daniels et al., 2006) and differential interference contrast microscopy (Wang et al., 2011b; Zhang et al., 2011, 2010), and allow the measurement of calcium dipicolinic acid release from individual spores, giving unique information on the early events of the germination process. One disadvantage of these methods is that they do not allow measurements of individual spores over longer time frames. Alternative methods exist that allow monitoring spore germination and outgrowth until the first cell divisions. These methods are based on (semi-) automated time-lapse phase-contrast microscopy linked to image analysis systems allowing quantification of heterogeneity in the different phases of sporulation, germination and outgrowth as used previously to study outgrowth of *C. botulinum* spores (Stringer et al., 2005, 2009; Webb et al., 2007) and sporulation or outgrowth of *B. subtilis* (de Jong et al., 2010; Pandey et al., 2013; Veening et al., 2008).

The methods described above are useful for studying germination or actively dividing vegetative cells, but the phase between germination and outgrowth to a fully developed cell under preservation-like stress conditions is not covered. Recently, a direct imaging method has been developed that allows monitoring the growth of individual cells until the micro-colony stage (Den Besten et al., 2007; 2010b; Ingham et al.,2005; 2007). This method is based on culturing cells upon porous aluminium oxide strips, called Anopore. These strips are highly porous, can be placed on a nutrient agar base and will retain microorganisms on their surface, while also allowing liquid from the nutrient agar to reach the spores via the capillary action of the pores. The thin layer of liquid that covers the strip allows bacteria to divide. The nature of the strips allows harvest of the developing microcolonies from the nutrient agar, without disturbing the microcolony structure. These strips can be placed on microscope slides covered with agar, containing a fluorescent dye to stain the cells of the microcolony for visualization with fluorescence microscopy. This approach has successfully been used to study population heterogeneity of *B. cereus* (Den Besten et al., 2007, 2010b) and *Lactobacillus plantarum* (Ingham et al., 2005, 2007) cells. This methodology can be adopted to study germination and outgrowth of a dormant

PhD Thesis Clint van Melis_corrected1.indd 21 4-12-2013 10:55:41

spore population, starting from the outgrowing stage to 8th generation microcolonies consisting of up to 256 cells.

3. Thesis outline

This project was initiated to assess the mechanisms underlying *B. cereus* spore germination and outgrowth in the absence and presence of food preservation stresses. This information can be of relevance to the food industry, as a better understanding of germination behavior under stress conditions relevant for food preservation may contribute to the development of new or improved concepts for enhanced control of spore forming bacteria in foods. Moreover, studying spore germination and outgrowth under stress conditions may improve our understanding of outgrowth heterogeneity and the impact of food preservation hurdles therein. The results described in this thesis represent the studies performed to understand spore germination and outgrowth under stressful conditions. Chapter 1 provides an introduction to the current knowledge on bacterial spores and the challenges for the food industry. It provides an introduction to *B. cereus* ATCC 14579 which is used as a model organism in this project and provides an overview of the stresses that are applied in the food industry and their presumed mode of action on bacteria. Finally, it explains why outgrowth heterogeneity causes problems in preservation and it gives an overview of techniques that can be used to study (heterogeneity in) outgrowth of bacterial spores.

The project aim was to provide insights in *B. cereus* spore germination and outgrowth under sorbic acid and other mild preservation stresses. The impact of the use of mild stresses on germination- and outgrowth heterogeneity was studied both on population- and single-cell level. The project was started by studying germination and outgrowth of *B. cereus* spores in the presence of sorbic acid, a common control agent used by the food industry. The inhibitory effects of undissociated sorbic acid were monitored both at phenotype and transcriptome level. The observed phenotypes and the corresponding transcriptome analyses are described in Chapter 2. The phenotypes and corresponding transcriptomes that were observed in the presence of higher concentrations of sorbic acid triggered us to study the mode of action of undissociated sorbic acid in spores. Chapter 3 describes the results of a series of experiments that were performed to identify the target of sorbic acid in the germinating spore. This study enabled us to pinpoint the interference site of high concentrations of undissociated sorbic acid in the germination pathway. This led us to question to what extend the inhibitory effect of organic acids on spore germination is determined by their lipophilic properties. To answer this question, a range of organic acids and structurally similar alcohols were evaluated for their lipophilic behavior and their corresponding germination-inhibiting properties. This study is described in Chapter 4.

The second aspect of spore outgrowth focused on in this thesis project was the heterogeneity inherent in outgrowth of a spore population and the influence preservation-stresses have on it. The outgrowth heterogeneity, either inherent or stress-induced was studied at both single cell level and population level. In the single cell approach described in Chapter 5, single, dormant spores were individually sorted using flow cytometry and their outgrowth behavior was monitored under neutral and (organic-) acid-stressed outgrowth. In the second approach, a population of individual spores was followed during germination and the first number of cell divisions

PhD Thesis Clint van Melis_corrected1.indd 22 4-12-2013 10:55:41

under a range of preservation-like stress conditions. The effects of mild pH-, salt-, organic acid-, and heat-stress on the development of microcolonies were visualized with a fluorescence based direct-imaging approach combined with Anopore technology. This work is described in Chapter 6. Finally, a summarizing discussion, concluding remarks, and future perspectives of the research described in this thesis are presented in Chapter 7.

PhD Thesis Clint van Melis_corrected1.indd 23 4-12-2013 10:55:41

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PhD Thesis Clint van Melis_corrected1.indd 37 4-12-2013 10:55:42

PhD Thesis Clint van Melis_corrected1.indd 38 4-12-2013 10:55:42

Chapter 2

Characterization of germination and outgrowth of sorbic acid-stressed *Bacillus cereus* ATCC 14579 spores: phenotype and transcriptome analysis

C.C.J. van Melis, M.N. Nierop Groot, M.H. Tempelaars, R. Moezelaar, and T. Abee

Published in Food Microbiology (2011), vol. 28(2):275-283

PhD Thesis Clint van Melis_corrected1.indd 39 4-12-2013 10:55:42

Sorbic acid (SA) is widely used as a preservative, but the effect of SA on spore germination and outgrowth has gained limited attention up to now. Therefore, the effect of sorbic acid on germination of spores of *Bacillus cereus* strain ATCC 14579 was analyzed both at phenotype and transcriptome level. Spore germination and outgrowth were assessed at pH 5.5 without and with 0.75, 1.5 and 3.0mM (final concentrations) undissociated sorbic acid (HSA). This resulted in distinct HSA concentration-dependent phenotypes, varying from reduced germination and outgrowth rates to complete blockage of germination at 3.0mM HSA. The phenotypes reflecting different stages in the germination process could be confirmed using flow cytometry and could be recognized at transcriptome level by distinct expression profiles. In the absence and presence of 0.75 and 1.5mM HSA, similar cellular ATP levels were found up to the initial stage of outgrowth, suggesting that HSA-induced inhibition of outgrowth is not caused by depletion of ATP. Transcriptome analysis revealed the presence of a limited number of transcripts in dormant spores, outgrowth related expression, and genes specifically associated with sorbic acid stress, including alterations in cell envelope and multi-drug resistance. The potential role of these HSA-stress associated genes in spore outgrowth is discussed.

Introduction

B. cereus is a Gram-positive, spore-forming soil bacterium that is widespread in nature. *B. cereus* is commonly encountered in the food-processing industry because of its highly adhesive spores, causing food-borne illnesses (Stenfors Arnesen et al. 2008) and premature spoilage of food products (Andersson et al. 1995). Although the effects of food-borne illness caused by *B. cereus* are generally mild, it is one of the most frequent causative agents (Stenfors Arnesen et al. 2008). Thermal processes necessary to eliminate spores from pathogenic and heat resistant spoilage microorganisms negatively affect organoleptic properties of food products. Currently, there is a tendency by consumer and food industry to desire milder processing conditions that allow higher quality foods (Abee and Wouters 1999).

In the food industry's search for more effective and less intense preservation as a basis for product innovation, spores including those of *B. cereus* are considered a major problem. The application of milder heating regimes is only feasible in combination with addition of preservatives to food-products to ensure safety and prevent spoilage. Weak organic acids are the most commonly used chemical preservatives in the food industry (Theron and Lues 2007). The inhibition by weak acids is based on the rapid diffusion of undissociated molecules through the plasma membrane, followed by immediate dissociation and acidification of the cytoplasm (Brul and Coote 1999; Lambert and Stratford 1999). Sorbic acid (SA) is among the most effective organic acids against a large group of (spoilage) organisms, including bacteria, yeasts and molds (Beales 2004). The use of sorbic acid is restricted to a subset of food products and is regulated via the European Directive for food additives (No. 95/29EC). For example, for emulsified sauces 1000 up to 2000 mg/kg is used. This corresponds with a range of 0.9 to 1.8mM HSA at pH 5.5. In addition to its impact on intracellular pH homeostasis, sorbic acid may have

PhD Thesis Clint van Melis_corrected1.indd 40 4-12-2013 10:55:43

(an) additional effect(s) on cellular performance, which can partly be explained by its relative high lipophilicity compared to other weak acids (Abbott et al. 2007), but the exact mechanism is still largely unknown.

We investigated the effect of sorbic acid on germination and outgrowth of *B. cereus* ATCC 14579 spores under mildly acid-stressed conditions. The effects on phenotype level were studied with spore germination assays, flow cytometry and measurements of ATP-concentrations. DNA microarray technology was used to study SA-induced effects on transcriptome level. Our results show that SA induces delayed germination and outgrowth of *B. cereus* spores. Transcriptome analysis revealed the presence of transcripts in dormant spores, genes related to outgrowth, and genes specifically associated with sorbic acid stress.

Materials and methods

Strain and culture conditions:

Bacillus cereus strain ATCC 14579 was obtained from the American Type Culture Collection (ATCC) and stored in Brain Heart Infusion (BHI) broth supplemented with 50% glycerol at -80°C. Unless stated otherwise, *B. cereus* was cultivated at 30°C with aeration at 200 rpm (Innova 4335 Incubator Shaker, New Brunswick Scientific, United States). Spores of *B. cereus* were harvested from cultures grown in defined, minimal sporulation medium described previously (de Vries et al. 2004). Five hundred µl of an overnight-grown pre-culture in BHI was used to inoculate 50ml minimal sporulation medium in 500ml flasks. Sporulation was followed by examining samples by phase contrast microscopy and by measuring the OD at 600 nm (OD600nm). Spores were harvested when >99% of the culture consisted of free spores (typically between 2 and 3 days). Spores were harvested by centrifugation at 5,000 rpm at 4°C for 15 minutes (Eppendorf centrifuge 5804 R, Eppendorf, Germany) and were washed three times with chilled phosphate buffered saline (PBS, 100mM, pH 7.1), containing 0.1% Tween 80 to prevent clotting of the spores. The spore solution was subsequently washed twice a day for two weeks to remove the remaining vegetative cells and germinated spores using PBS, supplemented with Tween 80 concentrations that decreased to 0.05%, 0.02% and 0% on day one, two and three, respectively. Pure spore crops devoid of vegetative cells and debris were stored in PBS at 4°C for not more than 8 weeks until use.

Spore germination assay

Spore germination was assessed under either unstressed conditions (BHI, pH 7.1), or under mild acid stress (BHI, pH 5.5, or BHI, pH 5.5 supplemented with SA). Spore germination was followed by the transition of phase bright spores turning phase dark, which is observed as a drop in optical density in a spectrophotometer (VersaMax , Molecular Devices, United States) as described previously (Hornstra et al. 2005). Spore density was adjusted in all experiments to an OD600nm of 0.8 – 0.9 to ensure that similar numbers of spores were used in all experiments (final concentrations of $2 \bullet 10^8$ cfu/ml). Prior to initiation of germination, spores were heat-activated for 10 min at 70°C, pelleted by centrifugation at maximum speed for 30 s (Eppendorf tabletop centrifuge, Germany) and washed and recovered in PBS. Twenty µl of spore solution was transferred to 250 µl wells in pre-cooled microtiter plates, followed by 180 µl of 1.1x concentrated BHI buffered with 100mM phosphate buffer at pH 7.1 (bBHI, pH 7.1). For

PhD Thesis Clint van Melis_corrected1.indd 41 4-12-2013 10:55:43

germination under mild acid stress, SA was added to BHI to obtain concentrations of 0, 0.75, 1.5 and 3.0mM undissociated sorbic acid (HSA), after adjusting the pH to 5.5. The OD600nm was measured every 30 s for 8 h in a VersaMax tuneable OD reader (Molecular Devices, United States) that was pre-warmed to 30°C. For each condition 2 independent biological replicates were used. Each replicate was repeated 8 times (technical replicates) resulting in 16 data points per condition.

ATP measurements

ATP measurements were performed with an LUMAC Biocounter M2500 using the ATP bioluminescence assay kit HS II (Roche, Germany). A standard curve of ATP (25pM – 100nM) was used to determine the unknown ATP concentration in the samples. Luciferase activity was found to be highly dependent on pH and temperature, consequently, all samples were adjusted to pH 7.8 using NaOH. Total ATP was determined by collecting 400 µl of sample into a tube containing beads (Lysing matrix B, MP Biomedicals, United Kingdom), NaOH (for pH correction) and an equal volume of lysis solution (kit ingredient) for direct inactivation. ATP background measurements were obtained by centrifugation (1 minute at maximum speed) of 500 µl sample aliquots after which 400 µl of supernatant was collected, pH corrected and an equal amount of lysis solution added. Samples were stored at -20°C until analysis. Total ATP samples were lysed by bead beating for 2 rounds of 1 min. Samples were diluted using the dilution buffer provided by the ATP kit to obtain ATP concentrations that fitted within the linear part of the standard curve. Intracellular total ATP levels were calculated by subtracting background ATP levels from total ATP levels. In the same experiment, samples were plated on BHI agar plates to obtain total viable counts which were used to determine the amount of ATP present per spore or cell.

Flow cytometry

Spore germination and outgrowth was followed by flow cytometric (FCM) analysis using the the fluorescent reporter dye SYTO-9 (Invitrogen, The Netherlands) which fluoresces only when bound to either double-stranded DNA or RNA with an emission maximum of 520 nm (green). Dormant spores show only peripheral staining with SYTO9, however, intact germinating spores stain brightly (Cronin and Wilkinson 2007).

Preliminary results showed that spore germination was completely blocked in the presence of 3mM HSA and this effect was used to arrest spore germination during sample handling for further FCM analysis. To this end, PBS (pH 5.5) was supplemented with SA to a final concentration of 3mM HSA and SYTO9 was added to a final concentration of 1µM. Samples were taken for FCM analysis after 10, 30, 60 and 120 min. These samples were prepared by centrifugation (maximum speed, 30 s) of 1 ml of culture and resuspension of the resulting pellet in 1 ml filtered PBS (pH 5.5), that was supplemented with 3mM HSA. Samples were incubated with the fluorescent dye for 15 min before measurement on a Becton Dickinson FACSCalibur flow cytometer with the following photomultiplier tube (PMT) voltage settings: E00 (FSC), 360 (SSC) and 690 (FL1). Data were obtained for 50,000 events (spores and/or cells) using Cellquest Pro (version 4.0.2), subsequently analyzed with WinMDI 2.9 (Joseph Trotter, Salk Institute for Biological Studies, La Jolla, California, USA; http://facs.scripps.edu/software.html).

PhD Thesis Clint van Melis_corrected1.indd 42 4-12-2013 10:55:43

Sampling and RNA isolation for microarray experiments

RNA was isolated from dormant, germinating and outgrowing spores in bBHI (pH 5.5) containing either 0, 0.75 or 1.5mM undissociated sorbic acid. Cultures were prepared by inoculating 20ml of the appropriate medium with heat-activated spores to reach a final concentration of $1 \bullet 10^8$ spores/ml. RNA was isolated from both dormant spores (t0) and germinating/outgrowing spores 10, 30, 60 and 120 min after initiation of germination. Twenty ml samples were rapidly pelleted at maximum speed at 4°C for 30 seconds. The pellet was resuspended in 1 ml TRI-reagent (Applied Biosystems, United Kingdom) and rapidly frozen in liquid nitrogen. The samples were stored at -80°C until RNA extraction.

For the extraction of RNA, the protocol previously described (van Schaik et al. 2004) was used with the modification that Lysing Matrix B beads (MP Biomedicals, Germany) were used. Residual chromosomal DNA was removed by treating the samples with DNA-free (Applied Biosystems, United Kingdom). The RNA quality and quantity were checked by UV spectroscopy (Biophotometer, Eppendorf, Germany) and by analysis on a RNA 6000 Nanochip (Agilent, United States). The RNA samples were stored in 70% ethanol with 83mM sodium acetate buffer (pH5.2) at -20°C.

cDNA synthesis, labelling and microarray hybridization and design

Fluorescently-labelled cDNA was prepared from the extracted RNA following an indirect labelling approach with amino-allyl-labelled dUTP (Ambion, United Kingdom) and Superscript III (Invitrogen, The Netherlands) as described previously (van Schaik et al. 2007). For every time point, independent biological duplicates were used and hybridized to the microarrays in a dye-swap approach (Cy3- and Cy5-labelling). For the microarray hybridizations, ~200 ng Cy3 or Cy5-labelled cDNA prepared from dormant spores (t0) was hybridized with cDNA prepared from germinating and outgrowing spores (1:1 ratio) after 10, 30, 60 and 120 min. Hybridization of the slides and removal of the unbound cDNA was performed following the 8 x 15K microarray processing protocol (Agilent, United States), with the following modification: the slides were washed with washbuffer 2 (0.1x SSC with 0.005% Triton X-102) for 5 min at 4°C, followed by the same procedure with pre-heated washbuffer 2 at 37°C.

The microarrays used in this study were custom-made *B. cereus* ATCC 14579 microarrays and were based on the 8 x 15K platform of Agilent. They comprised a total of 10,704 probes representing 5249 chromosomal open reading frames, 21 plasmid-encoded open reading frames and 82 putative small-RNAs, meaning that 97.6% of the predicted chromosomal open reading frames (NCBI accession number NC_0044722) are represented on the microarray.

Microarray scanning and data analysis

The microarray slides were scanned using an Agilent microarray scanner (G2565BA), and raw data were extracted using Agilent's Feature Extraction software (version 8.1.1.1). A considerable bias was observed when data normalization was performed using the spot intensities of Cy3 and Cy5 channels (dormant t0 versus time point sample) on the original array. Therefore, microarrays were normalized using the following approach: the background-corrected, raw signals (Cy3 and Cy5 channel) of all arrays were hierarchically clustered (Pearson correlation,

PhD Thesis Clint van Melis_corrected1.indd 43 4-12-2013 10:55:43

complete linkage) using Genemaths XT (version 1.6.1, Applied Maths, Belgium). New synthetic arrays were defined from sample pairs showing the highest similarity in the clustering and consisted of a Cy3- and a Cy5-dyed sample. In a next step, the synthetic microarrays were Lowess normalized using the MicroPrep software package (version 8) (van Hijum et al. 2003). A feature was found to be differentially expressed when both spots representing this feature were significantly and differentially expressed between samples, with a false discovery rate (FDR) smaller than 0.05. Genes for which no significant values were obtained for any of the conditions were omitted from the dataset. MultiExperiment Viewer (version 4.3; TM4 microarray software suite; http://www.tm4.org/mev.html) was used to identify spore- and phenotype-specific transcripts by means of K-means clustering. A selection of 20 clusters was used for a rough identification of phenotype- or stress-specific responses, and was refined by selecting only genes that were up- or down-regulated at least twice in one or more of the tested conditions.

Microarray accession number

Microarray data are deposited in the GEO database (http://www.ncbi.nlm.gov/geo/) under accession number GSE19186.

Results

Impact of sorbic acid on germination and outgrowth

To assess the impact of SA on germination, BHI was supplemented with SA resulting in final concentrations of 0.75, 1.5 and 3.0mM HSA. Germination was followed by monitoring the relative drop in optical density at 600 nm (OD600nm) as described in the materials and methods section. Germination at pH 7.1 (referred to as 'unstressed germination' in this manuscript) was used as a reference to germination of spores without pH stress.

Within 15 min, the OD_{600}-values of the unstressed spore cultures were reduced to approximately 40% of the initial optical density value (OD t=0, Fig. 1). This drop in OD represents close to 100% germination, as visualized by phase contrast microscopy (Fig. 2, panel B). The drop in OD results from dormant spores taking up water and thereby losing refractability, which appears as phase bright spores turning phase dark as revealed by phase contrast microscopy. The maximum drop in OD was immediately followed by an increase in optical density as a result of spore outgrowth in BHI (pH 7.1). Germination of spores under mild acid stress (pH 5.5, no SA) was slightly delayed compared to germination at pH 7.1, reaching complete germination after approximately 30 min (Fig. 1). With increasing concentrations of HSA, the maximum drop in optical density decreased; reaching 50% (0.75mM HSA) or 60% (1.5mM) of the ODt=0, whereas at 3mM HSA germination was completely blocked although this effect could be relieved when transferred to SA-free media (data not shown). Both for the mildly acid-stressed conditions with (0.75mM) and without SA (0mM) at pH 5.5, spores were able to grow out, albeit at a lower rate compared to pH 7.1. Spores exposed to 1.5mM HSA did germinate, but failed to grow out under this condition. Spores exposed to 3mM HSA did not show the same type of OD-drop. The limited and gradual decrease in OD noted for the spores exposed to 3mM HSA appeared not to be associated with germination, as phase contrast microscopy only revealed the presence of dormant phase bright spores.

PhD Thesis Clint van Melis_corrected1.indd 44 4-12-2013 10:55:43

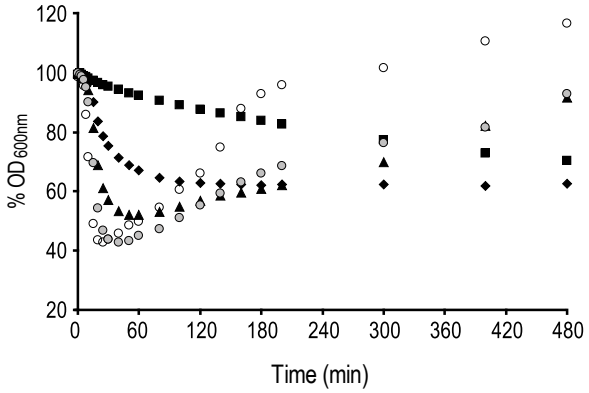


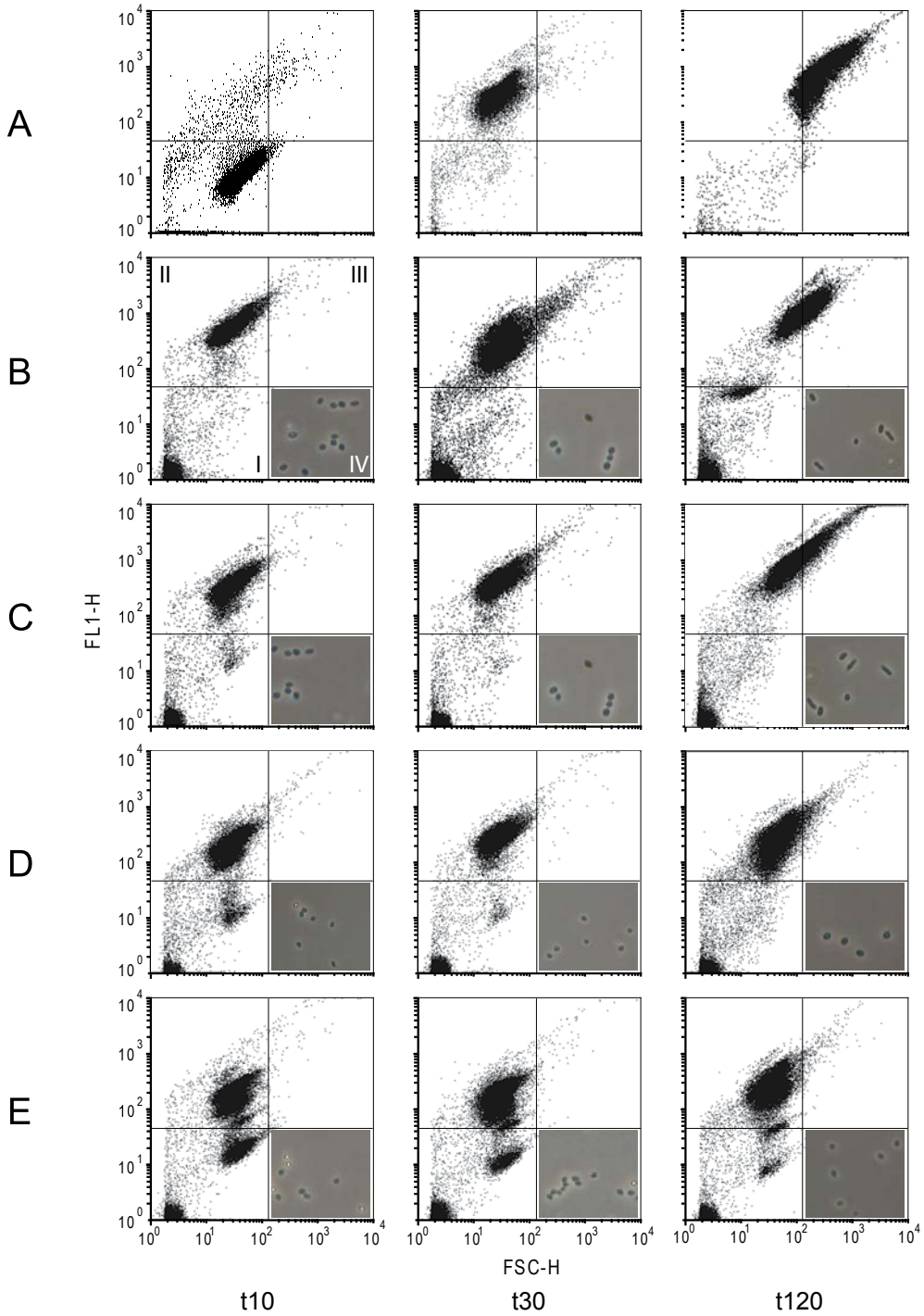
Fig. 1: Germination and outgrowth of *B. cereus* spores. Germination and outgrowth was followed in time by the transitions of phase bright (dormant) spores to phase dark (germinated spores) by the change in optical density. Spores were either germinated at pH 7.1 (clear circles), at pH 5.5 without added SA (grey circles) or with 0.75mM (black triangles), 1.5mM (black diamonds) or 3.0mM (black squares) HSA. The y-axis shows the change in optical density (OD) relative to the OD at initiation of germination.

Flow cytometry analysis

Germination of heat-activated *B. cereus* ATCC 14579 spores is a largely homogeneous process under unstressed conditions. However, the reduced germination capacity and microscopy analysis suggested that the germination process occurs more heterogeneously in the presence of sorbic acid. To study this heterogeneity and the delay in germination under sorbic acid stress in more detail, spores were exposed to similar conditions as described above and their germination was followed by flow cytometry (Fig. 2). The position in the quadrants for each phase in germination and outgrowth was validated by staining a pure dormant spore culture as well as fully germinated spores and vegetative cells (Fig. 2A). Quadrant I was populated with slightly stained dormant spores. This quadrant also contained debris from the medium positioned in the left bottom corner. Upon spore germination, permeability to SYTO9 and accessibility of DNA increased which could be monitored by an increase in fluorescence level (quadrant II became populated; Fig. 2A). Outgrowth of germinated spores could be followed by an accumulation of events in quadrant III; an increase in forward-scatter signal represented the increase in particle size during the outgrowth phase.

After 15 min, for all conditions (except 3mM HSA, data not shown) at least part of the spore-populations became permeable to SYTO9 which coincided with the appearance of fluorescent particles in quadrant II. Under unstressed conditions (pH 7.1) the majority of the spore population was located in quadrant II within 10 min, while for the acid-stressed spores a substantial part of the spore population was located in quadrant I. A substantial part of the spores that germinated at pH 5.5 without HSA was located in quadrant III after 120 min, whereas sorbic acid-exposed spores dominated quadrant II. These observations confirmed that HSA-germinated spores display a delay in outgrowth compared to unstressed or mildly-stressed (pH 5.5, no HSA) spores. Phase contrast microscopy images of the corresponding samples (inset IV in Fig. 2) confirmed the observations made by FCM. Remarkably, the presence of various subpopulations of germinating spores could clearly be determined for 1.5mM HSA spores (panel E at t10), which may represent spores in different stages of germination. Clearly, cell elongation

PhD Thesis Clint van Melis_corrected1.indd 45 4-12-2013 10:55:43



PhD Thesis Clint van Melis_corrected1.indd 46 4-12-2013 10:55:49

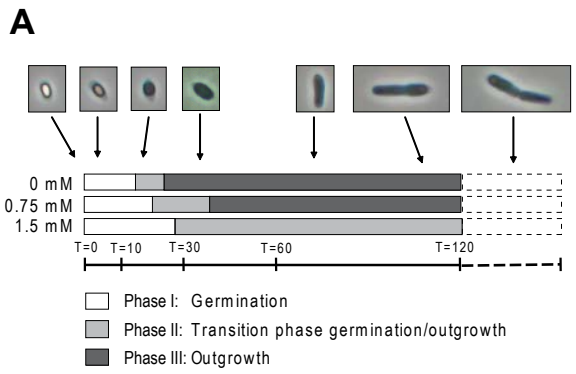
was visualized as elongated cells appeared after 120 min at pH 7.1 and pH 5.5 (no SA) (Fig. 2, panels B and C, quadrant III and inset IV). These cells were not observed in the conditions where SA was present (panels D and E of Fig. 2).

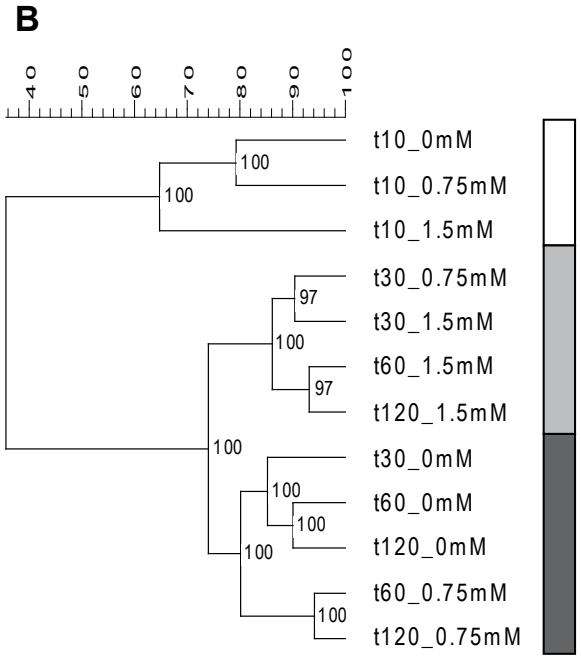
Transcriptional analysis of germinating and outgrowing spores under (mild) acid stress
DNA microarray analysis was performed to get more insight into the cellular processes that underlie the observed germination- and outgrowth behavior under sorbic acid stress. To this end, dormant spores were heat-activated and germinated in BHI at pH 5.5 with and without 0.75mM or 1.5mM HSA. Spore germination at pH 5.5 without HSA (0mM) was included as a control for pH 5.5-induced changes in gene expression, thus allowing for the identification of SA-specific responses.

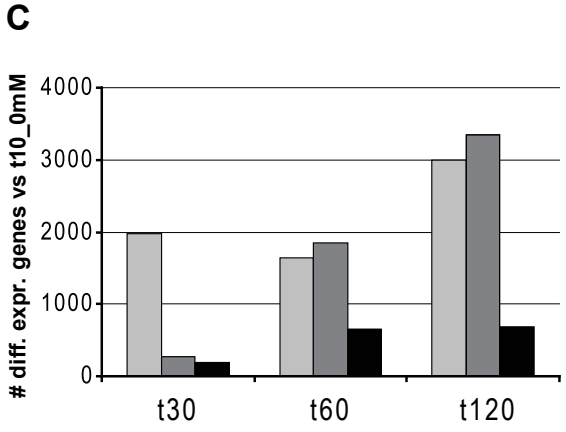
Hierarchic clustering of the transcriptome profiles of germinating spores revealed the existence of three distinct clusters, which corresponded with the observed germination- and outgrowth-phases (Fig. 3A and B). Ten minutes after the initiation of germination, spores were germinating under all conditions (Fig. 3A) and this phase coincided with a distinct gene expression profile (white cluster, Fig. 3B). Expression profiles in the second cluster (light grey, Fig. 3B) represent spores that are in a transition phase between germination and outgrowth. Phenotypically, spores included in this cluster showed maximum loss of refractability prior to the subsequent increase in OD600 associated with cell growth. By FCM, this phase could be characterized as spores that reach maximum fluorescence and occupy quadrant II of Figure 2. The third cluster (dark grey, Fig. 3B) represents expression profiles of outgrowing spores that coincided with a movement from quadrant II to III in the FCM experiments. Expression profiles of 1.5mM HSA-exposed spores were not represented in the last cluster, which is in line with the failure to grow out under these conditions. This suggests that in the presence of 1.5mM HSA genes related to the outgrowth phase of spore germination showed a different response compared to spores with outgrowth capacity. The 0.75mM exposed spores moved to the last cluster (dark grey) after 60 min, whereas spores that were not exposed to SA (0mM) already appeared in this cluster after 30 minutes. This corresponds with the delay in outgrowth for HSA-exposed spores that was observed at phenotype level. The energy status of the germinating spores was followed by measuring the intracellular concentrations of ATP at the above-mentioned conditions and time points (Fig. 4). For spores germinated without HSA, intracelluar ATP levels increased rapidly 60 min upon initiation of germination whereas for spores exposed to 0.75mM HAS, intracelluar ATP levels did not increase significantly until 120 min. For 1.5mM HSA-stressed spores, an increase in intracellular ATP level was observed at t120, albeit at a reduced rate compared to the spores that were exposed to less severe HSA-stress. This suggests that ATP generation was delayed, rather than impaired, under sorbic acid stress.

Fig. 2: FCM-derived scatterplots of green fluorescence (FL1–H) versus forward-scatter intensities (FSC-H) of SYTO 9 stained *B. cereus* spores during germination (t10 + t30) and outgrowth (t120). An increase in fluorescence level indicates the transition from dormant spore to fluorescent germinated spore and increase in forward-scatter signal relates to an increase in particle size. Panel A) from left to right: Dormant spores, germinating spores and vegetative cells, respectively. Panels B-E) Spores were germinated at pH 7.1 (B), at pH 5.5 without the addition of sorbic acid (C) and pH 5.5 with 0.75mM (D) and 1.5mM HSA (E). Events in different quadrants correspond to dormant spores (I), germinated spores (II) and vegetative cells (III). In quadrant IV, images, obtained by phase contrast microscopy, are shown for the various conditions.

PhD Thesis Clint van Melis_corrected1.indd 47 4-12-2013 10:55:50







PhD Thesis Clint van Melis_corrected1.indd 48 4-12-2013 10:55:50

To visualize outgrowth related gene expression more clearly, the number of differentially expressed genes (both up- and down-regulated) for each time point was compared to spores not exposed to sorbic acid (0mM SA) during germination at t10 (Fig. 3C). The t10 sampling point of 0mM HSA was chosen as the point marking the transition from germination to outgrowth for mildly-stressed spores. The number of genes that are differentially expressed compared to t10 (0mM HSA) for both sorbic acid conditions is smallest at t30, indicating that the transcription profiles at this sampling point were most comparable to those of mildly-stressed spores after 10 min. Differences between the two HSA-induced phenotypes became more pronounced later in the germination process when the germinated spores initiated outgrowth. After 60 and 120 min the number of significantly expressed genes continued to increase in spores with the outgrowth phenotype (0mM and 0.75mM HSA), while the number of differentially expressed genes in spores that did not show outgrowth (1.5mM HSA) remained at approximately the same level.

Spore transcripts

In a first attempt to classify patterns of gene expression upon spore germination, a K-means clustering analysis was performed. The large majority of the genes present on the microarrays was significantly up-regulated under most conditions (e.g. ~80% of the genes was up-regulated at t120 for 0.75mM HSA) which did not result in clear patterns that allowed for the identification of subsets of genes related to specific phenotypic responses. However, this clustering analysis (see methods) revealed a small subset of 46 genes that displayed a striking rapid downward trend in expression levels in time for the 0mM dataset (Fig. 5). These transcripts were abundantly present in the dormant spore, but were rapidly broken down upon the onset of germination. This cluster included genes associated with spore coat composition (cotJA, spore coat proteins K and M), small acid-soluble proteins (SASP's), spore germination protein GerD and a large number of open reading frames encoding hypothetical proteins. Eight of these genes are known to be regulated by the sigma factor SigmaG in *B. subtilis* (see table S1 in the supplementary data for details). Interestingly, the spore transcripts were not rapidly broken down in the spores that were exposed to 0.75mM and 1.5mM HSA despite the fact that these spore populations consisted largely of germinated spores.

Genes related to the outgrowth phenotype

To identify outgrowth related genes, genes were selected that displayed an expression pattern meeting the following constraints: 1) genes should be up-regulated at least two-fold at t60 and

Fig. 3: Sorbic acid-induced changes at phenotype and transcriptome level of *B. cereus* ATCC 14579 spores upon germination and outgrowth. A) Schematic representation of different germination- and outgrowth-phases at medium pH 5.5 without SA (0mM) or in the presence of 0.75 or 1.5 mM HSA. Photographs of individual spores illustrate the physiological state of the spores. B) Hierarchical clustering of gene expression profiles of spores germinated at pH 5.5 without (0 mM) or with addition of sorbic acid to 0.75 or 1.5 mM HSA. Clusters were obtained by using the complete linkage algorithm combined with Pearson's correlation for the microarrays for each condition and sampling time point. Indicated values represent bootstrapped values. C) The number of genes that were differentially expressed (up or down) under sorbic acid stress compared to germinating spores at pH 5.5 without sorbic acid (0mM at t10). Spores germinated under mild acid-stressed conditions at pH 5.5 with 0mM, 0.75mM and 1.5mM HSA are represented by light grey, dark grey and black bars, respectively. The pH for both sorbic acid concentrations was adjusted to a value of 5.5 by the addition of HCl. The t10 sampling point of 0 mM HSA was chosen as this point marks the transition from germination to outgrowth for mildly-stressed spores.

PhD Thesis Clint van Melis_corrected1.indd 49 4-12-2013 10:55:50

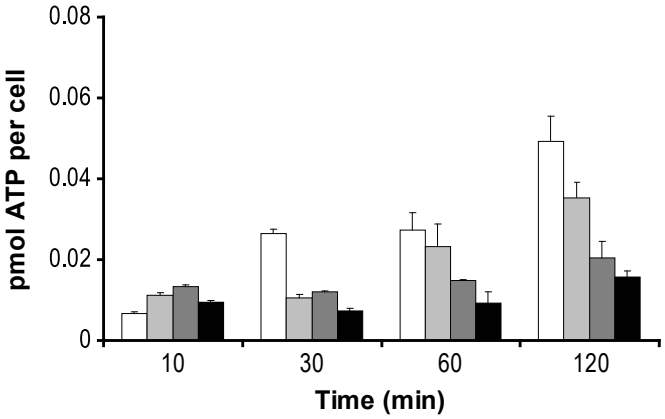


Fig. 4: ATP concentrations in *B. cereus* ATCC 14579 spores during germination and outgrowth. Spores germinated under non-stressed conditions at pH 7.1 (clear bars) and under stressed conditions at pH 5.5 with either 0mM HSA (light grey bars), 0.75mM HSA (dark grey bars) or 1.5mM HSA (black bars). The pH for both HSA concentrations was adjusted to a value of 5.5 by the addition of HCl.

t120 in 0mM and 0.75mM and 2) should not be up-regulated in the presence of 1.5mM HSA. This resulted in a subset of 704 genes that were only up-regulated in spores that displayed the outgrowth phenotype (0mM and 0.75mM HSA) of which approximately 30% of the genes encoded for hypothetical proteins. The majority of these genes were expressed immediately after germination (t10 and t30 for non-HSA and HSA-exposed spores, respectively) and increased during the later time points, or remained on approximately the same level of expression. About a third of the genes (704 genes) was only expressed after 120 minutes. Among these 704 genes, 54 genes were involved in transcription; 33 genes encoding transcriptional regulators were identified, including PlcR (BC5350) with a role in the expression of virulence factors in *B. cereus*, and the sigma factors *sigW* (BC5363), *sigF* (BC2108), *sigZ* (BC2108) and RNA polymerase sigma-70 factor (BC5251), and regulators such as the SigmaB regulator *rsbU* (BC1006), the anti-SigmaF antagonist BC4074 and a SigmaM negative effector (BC1113). Another transcriptional regulator displaying this expression pattern was BC2444 which shows similarity to the steady state regulator protein AbrB, although the level of the nearest ortholog of *B. subtilis*

AbrB (BC0042) and two other homologues (BC1884 and BC1996) were not significantly altered. Other large groups of genes could be classified into multidrug (MDR) transporters and cell envelope modification and synthesis. Among these transporters are two homologues of multidrug resistance protein B (BC1757 and BC2880) and a gene predicted to encode a multidrug resistance protein (BC4569), of which a homologue in *B. subtilis* codes for MDR protein A, a putative exporter protein.

Sorbic acid specific genes

To identify a sorbic acid specific response, genes were analyzed that were expressed in the presence of either 0.75mM, 1.5mM, or both concentrations of HSA and not in spores germinating at pH 5.5 without sorbic acid. This resulted in a set of 245 genes in which three main functional groups could be distinguished; cell envelope modifications, (multidrug) transporters and amino-acid metabolism (Fig. 6). The first group included genes such as a long chain fat-

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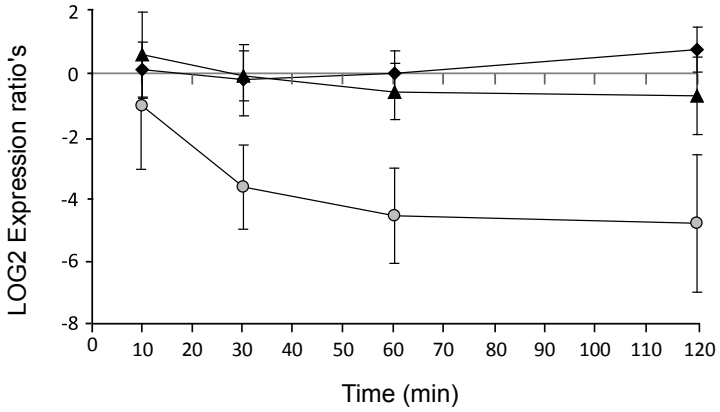

Fig. 5: Expression profiles (LOG2 values) of transcripts present in dormant spores. An average of the expression patterns for all genes is shown here as a representative for the 46 genes for which transcripts were identified. The expression patterns are shown for *B. cereus* ATCC 14579 spores that germinated at pH 5.5 without sorbic acid (light grey circles), 0.75mM HSA (black triangles) and 1.5 mM HSA (black diamonds).

ty acid acetyl-CoA ligase (BC3628), propionyl-CoA carboxylase (*yngE* (BC2488) and a lipase (BC4862), all known to be involved in the metabolism of fatty acids (Fig. 6A). Homologues of *B. subtilis* genes coding for penicillin-binding-protein 4 (*dacF*, BC4075 and BC3188) were specifically up-regulated when exposed to 0.75mM HSA. Genes with functions related to modifications of the cell surface, such as *cpsC* (BC1584) with predicted function in regulation of capsular polysaccharide biosynthesis and *srtC* (BC4811) encoding a sortase enzyme, also belong in the group of alterations to cell envelope. Approximately 10 percent of the SA-specific genes are involved in transport (Fig. 6B), such as (multi-)drug resistance proteins (BC2061, BC2681 and BC4537), transport of sugars (BC2960- BC2963 operon) and influx of oligopeptides (OppB homologues BC0908 and BC2326). Genes related to amino-acid metabolism that were up-regulated under sorbic acid stress were involved in the synthesis and transport of amino acids with high nitrogen-content, such as histidine (*hisD*, BC1406 and *hisD*, BC1407) and arginine (*rocC*, BC2980) (Fig.6C). Most genes required for the biosynthesis of the branched chain amino acids leucine and valine (BC1399 – BC1403) were also specifically up-regulated. Furthermore, the expression of a large group of genes, with a variety of putative functions, also differentially changed under these conditions (see table S2 in the supplementary data for an overview of SA-specific genes).

Discussion

In this study we provide a detailed analysis of the phenotypic and transcriptional responses linked to germination and outgrowth of *B. cereus* spores at pH 5.5 in the absence and presence of sorbic acid. Three concentration-dependent germination phenotypes were observed in the presence of sorbic acid. Spores exposed to 3mM HSA did not germinate at all during the experiments. In the presence of 0.75mM and 1.5mM HSAat pH 5.5, spores were capable of germination albeit at reduced rate compared to spores germinating in BHI of pH 5.5 without HSA, with spores growing out only at 0.75mM HSA. No direct evidence was found that the observed sorbic acid-induced delay in outgrowth is caused by a depletion of intracellular ATP, since

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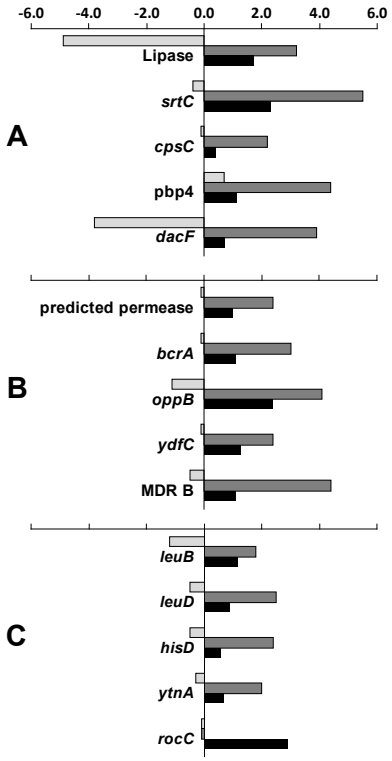

Fig. 6: Expression ratios (LOG2 values) of selected genes obtained from the sorbic acid-induced gene cluster expressed at t120. Values are shown for *B. cereus* ATCC 14579 spores that germinated at pH 5.5 without sorbic acid (light grey bars), 0.75mM HSA (dark grey bars) and 1.5 mM HSA (black bars). A selection of genes is shown for the overrepresented groups of cell envelope modifications (A), transporters (B) and amino-acid metabolism (C). The group of cell envelope modifications is represented by a lipase (BC4862), *srtC*, encoding a fimbria-associated sortase (BC4811), *cpsC*, which encodes capsular polysaccharide protein CpsC (BC1584), and *pbp4* and *dacF*, encoding homologues of *B. subtilis* penicillin-binding-protein 4 (BC3188 and BC4075). The group of transporters is represented by a predicted permease (BC0761), *bcrA*, which codes for an ATP-binding protein BcrA (BC4537) of a bacitracin transporter, *oppB*, which encodes an oligopeptide transport system permease protein (BC2326), *ydfC*, encoding a transporter belonging to a drug/metabolite exporter family (BC2681) and MDR B, which codes for a multidrug resistance protein B homologue (BC2061). The group of amino-acid metabolism is represented by *leuB* (BC1401) and *leuD* (BC1403), which encode for a 3-isopropylmalate dehydrogenase and dehydratase, respectively. The selection also includes a histidinol dehydrogenase, *hidD* (BC1406), *ytnA*, encoding a proline-specific permease (BC0688) and an arginine permease, encoded by *rocC* (BC2980).

similar ATP levels were found in populations of outgrowing 0.75mM HSA-exposed spores and non-outgrowing spores exposed to 1.5mM HSA.

An explanation for the reduced germination capacity could relate to the hydrophobicity of HSA, causing it to accumulate in the spore's inner membrane. This accumulation may interfere with the signaling cascade, which is required for germination (Moir, 2006). Previous research by others showed that triggering of nutrient-induced germination may be hampered by sorbic acid (Blocher and Busta 1985; Cortezzo et al. 2004; Smoot and Pierson 1981), but the exact mechanism of inhibition is not fully understood and is subject for further research.

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Phenotypic observations showed that mildly acid-stressed *B. cereus* spores germinate homogeneously in absence of sorbic acid, while 3mM HSA completely blocks germination. Spores exposed to HSA concentrations in the range between these extremes displayed subpopulations with different germination rates. The fluorescent DNA probe SYTO9 was previously used to distinguish germinating spores from dormant spores in end-point measurements (Cronin and Wilkinson 2007; Cronin and Wilkinson 2008; Laflamme et al. 2005). In our set-up we used the strong germination blocking activity of 3mM HSA to arrest the dormant spore fractions which allowed us to follow the germination process in a time course experiment. Using this FCM approach, we were able to provide a more detailed dissection of subpopulations occurring when spores germinate in the presence of SA. Studies concerning the existence and characteristics of subpopulations displaying heterogeneity in germination and outgrowth behavior are highly relevant, since specific subpopulations may show improved survival and/or outgrowth performance under preservation stress. In conclusion, information about factors contributing to heterogeneity in resistance and germination/outgrowth performance is of relevance for the food-industry, because this may allow for improvement of predictions on germination and outgrowth capacity of spores, thus in the end allowing better control of spore formers. Application of FCM and characterization of sorted sub-populations may contribute to further understanding of diversity and heterogeneity in performance of spore populations.

To get more insight in the cellular processes that underlie the observed phenotypes, microarray experiments were performed. Hierarchical clustering identified transcription profiles that corresponded with the observed germination- and outgrowth phenotypes. The majority of genes in the genome of *B. cereus* was expressed during spore germination and outgrowth, including most stress- and chemotaxis-related genes. SigmaB-related stress genes and SigmaD-related chemotaxis genes were also reported to play an important role in the outgrowth of *B. subtilis* spores (Keijser et al. 2007). Additionally, many genes involved in the response to oxidative stress were expressed directly after germination. These genes were also observed to be expressed in *B. cereus* vegetative cells, exposed to mild acid stressed conditions at pH 5.4 (Mols et al. 2010). Spores are triggered to germinate and initiate vegetative growth upon sensing the presence of favorable nutrients. From the data in the present study, it appeared that the early events in germination and outgrowth were triggered, irrespective of whether conditions for outgrowth were favorable or not. This is supported by the gene expression data, which showed that in the initial events of germination genes that are induced in the control and SA-stressed spores largely overlap. This is in line with observations made in *B. subtilis*, where detailed transcriptome analysis revealed spore germination to occur via a tightly-controlled spore outgrowth program (Keijser et al. 2007). Indeed, the *B. cereus* spore outgrowth program shows high similarity with that of *B. subtilis*.

In the past, it was generally believed that spores were devoid of significant levels of mRNA (Armstrong and Sueoka. 1968; Chambon et al. 1968; Doi and Igarashi 1964; Hansen et al. 1970). More recent studies have identified the presence of specific transcripts in spores of *B. subtilis* (Keijser et al. 2007) and *Clostridium novyi*-NT (Bettegowda et al. 2006). In *B. cereus* we identified 46 genes that appeared highly abundant in dormant spores, whereas 23 transcripts were found in *B. subtilis* and 50 transcripts in *C. novyi*-NT. Eight of these genes are known to

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be regulated by SigG in *B. subtilis*, and these genes are associated with spore coat composition, small acid-soluble proteins (SASPs) or were hypothetical proteins without a known function. Another SigG regulated gene with this expression pattern is *gerD* that encodes a protein involved in germination. In *B. subtilis*, this gene encodes an inner membrane located lipoprotein that becomes soluble after spore germination and is degraded during outgrowth (Pelczar and Setlow 2008). A number of RNAs in dormant *B. cereus* spores are encoded by genes that have homologues in *B. subtilis*, including *sspF*, spore coat protein K, some SASPs and several hypothetical proteins (Keijser et al. 2007). Interestingly, the spore transcripts were not degraded in germinating spores that were exposed to 0.75mM and 1.5mM HSA despite the fact that these spores were fully germinated, suggesting that the degradation of these transcripts is somehow impaired, whereas germination-associated RNAs are detected at the same time. The role of dormant spore transcripts remains to be studied in more detail, including the impact of sporulation history on the number and types of transcripts, and their role, if any, in germination and outgrowth capacity.

Transcriptome studies revealed approximately 12% of the genome to be up-regulated in outgrowing spores (0mM and 0.75mM HSA). The majority of the outgrowth-specific genes were expressed directly after germination, while approximately 33% of the genes was only up-regulated during the late outgrowth phase. The latter group of genes belongs to a broad range of cellular functions, including many transcription factors, (MDR) transporters and genes involved in the synthesis and modification of the cell envelope. This subset of genes represents features that are associated with the ability to grow out.

Further analysis revealed a set of 267 genes to be specifically induced by sorbic acid at pH 5.5. Within this set of genes, three main groups were identified; cell envelope, (multidrug) transporters and several genes involved in the metabolism of amino-acids. Modification of the cell wall and/or membrane composition may diminish the accumulation of SA in the cell membrane. Incorporation of branched chain fatty acids is a known response of bacteria to low temperature and salt (Kaan et al. 2002; Weber et al. 2001) and was reported to occur in *B. subtilis* vegetative cells in response to SA (Ter Beek et al. 2008).

Our results on germinating spores showed increased expression of an acetyl-CoA synthase (BC3628) and lipase encoding gene (BC4862) suggesting that modification of the membrane may occur under SA stress. Genes involved in cell envelope modifications were significantly expressed under SA conditions. These genes make up approximately 10% of the genes involved in the observed HSA–response. Although the cell wall is generally considered a porous structure and thus not forming a barrier for the entry of small molecules, the gene expression data suggest that SA exposure leads to an adjustment of the cell wall. Alterations in branching of peptidoglycan layers is suggested by the strong up-regulation of DacF, which is involved in crosslinking of peptidoglycan chains (Popham et al. 1999), and a penicillin-binding-protein 4 (Pbp4) homologue (BC3188), that has been associated with cell wall modification in *B. subtilis* under osmotic stress conditions (Palomino et al. 2009). The synthesis of peptidoglycan is essential for cell wall biosynthesis and incorrect crosslinking of peptidoglycan chains can influence outgrowth performance (Murray et al. 1998a; Murray et al. 1998b). Interestingly, modification

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of the cell wall was reported to occur in yeast cells exposed to hydrophobic weak organic acids such as SA (Abbott et al. 2007). Moreover, our transcriptome analysis identified genes encompassing the capsular polysaccharide (*cps*)-cluster to be strongly up-regulated under SA stress, whereas for the mildly acid-stressed conditions these genes were only partly up-regulated at low levels. Increased capsule production may thereby potentially form an additional protective layer, i.e., diffusion barrier, surrounding the cell. Furthermore, up-regulation of *srtC*, encoding a fimbrial associated sortase, was noted. Sortases are involved in anchoring proteins to the cell envelope of Gram-positive bacteria (Marraffini et al. 2006). Recently, *srt*-sortases were reported to be involved in the formation of pili in *B. cereus* (Budzik et al. 2007; Budzik et al. 2009). These data suggest that SA-stressed germinating spores activate a range of cell wall and membrane modulating activities. Whether such modifications are implemented and contribute to SA resistance remains to be elucidated.

Another interesting feature of the SA-induced response involves the transcription activation of genes encoding multidrug resistance (MDR) systems. *B. subtilis* vegetative cells displayed an SA-specific response involving the up-regulation of the major facilitator superfamily multidrug resistance transporter YhcA (Ter Beek et al. 2008). In outgrowing *B. cereus* spores, a homologue of this gene was expressed under all conditions, even when no sorbic acid was present. However, the differences in experimental set-up may affect specific responses. Furthermore, sixteen homologous genes coding for multidrug resistance proteins B are present in the genome of *B. cereus* ATCC 14579 and notably, all of these are expressed directly after germination in all conditions with the exception of only one (BC2061) that was expressed specifically in the presence of HSA, pointing to a putative function in HSA resistance. It has been predicted that a wide range of MDR-like systems of the *B. cereus* ATCC 14579 genome are under control of two component systems belonging to the OmpR-like family (de Been et al. 2006). However, our transcriptome data did not reveal SA-specific expression of the OmpR type regulators. Additional studies are required to elucidate the role of (specific) MDR systems in (weak) acid and/or HSA resistance of *B. cereus*.

In conclusion, our spore transcriptome analysis resulted in the identification of a set of genes associated with sorbic acid-stress, providing a first step in the elucidation of key factors contributing to spore germination and outgrowth under sorbic acid-stress conditions.

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Chapter 3

Impact of sorbic acid on germinant receptor-dependent and independent germination pathways in *Bacillus cereus*

C.C.J. van Melis, M.N. Nierop Groot, and T. Abee

Published in Applied and Environmental Microbiology (2011),
vol. 77(7):2552 – 2554

PhD Thesis Clint van Melis_corrected1.indd 59 4-12-2013 10:55:51

Amino acid and inosine-induced germination of *Bacillus cereus* ATCC 14579 spores was reversibly inhibited in the presence of 3mM undissociated sorbic acid. Exposure to high hydrostatic pressure, Ca-DPA, and bryostatin, an activator of PrkC kinase, negated this inhibition, pointing to specific blockage of signal-transduction in germinant receptor-mediated germination.

A wide range of food preservatives, including weak acids such as lactates and sorbic acid (SA), are effective in maintenance of food quality and safety (5, 14) and are therefore widely used in industry. We previously assessed the impact of SA on germination and outgrowth of *Bacillus cereus* spores (16) and reported that at concentrations of 3 mM undissociated sorbic acid (HSA) and higher, amino acid- and inosineinduced spore germination was blocked, with spores remaining fully refractile. An earlier study on *Clostridium botulinum* and *B. cereus* spores suggested that the mode of action of sorbic acid was by competitive inhibition on the L-alanine/inosine receptor (13). In later work performed by Blocher and Busta (3), competitive inhibition was disputed, and evidence was provided that led the authors to propose that inhibition occurred after germinant binding and could involve alteration of the spore's inner membrane permeability or inhibition of cortex lytic enzymes. Based on current knowledge, the *B. cereus* ATCC 14579 genome encodes seven putative germinant receptors, and spore germination in this organism can be triggered not only by alanine, via the germinant receptor GerR, but also by a range of other germinants, including inosine, adenosine, glutamine, cysteine, threonine, and phenylalanine (7).

This triggered us to assess the impact of sorbic acid on the different Ger receptors by using inosine and selected amino acids as germinants with both *B. cereus* ATCC 14579 wild-type spores and gerR deletion mutant spores, which are insensitive to alanine-induced germination (6). Spore preparations of the *B. cereus* ATCC 14579 wild type and its *gerR* deletion mutant were prepared for germination assays as described previously (6, 16). In short, heat-activated spores (70°C for 15 min) of both the wild type and *gerR* mutant were resuspended in morpholineethanesulfonic acid (MES) buffer corresponding to the test conditions prior to addition to a microtiter plate. Both wild-type and *gerR* mutant spores were exposed to a 1 mM concentration of one of the germinants L-alanine, L-glutamine, or inosine, thereby triggering GerR, GerG and GerQ, and GerI, respectively, as demonstrated by Hornstra et al. (7). The spores were incubated at pH 5.5 with or without 3 mM HSA and germinant (Fig. 1). To test the reversibility of HSA-arrested germination, the spores were incubated for 2 h in the presence of selected germinants before the pH was raised to 7.1 by the addition of NaOH in the HSA-exposed samples. This instantaneously shifted the equilibrium between SA and HSA, resulting in a decrease from 3 mM HSA to less than 0.2 mM, with a concomitant relief of inhibition of germination (Fig. 1).

The data show that not only germination via L-alanine but also that via the germinants inosine and glutamine is inhibited by the presence of 3 mM HSA. Comparable results were obtained for germination induced with cysteine, threonine, and phenylalanine (data not shown). Thus, HSA

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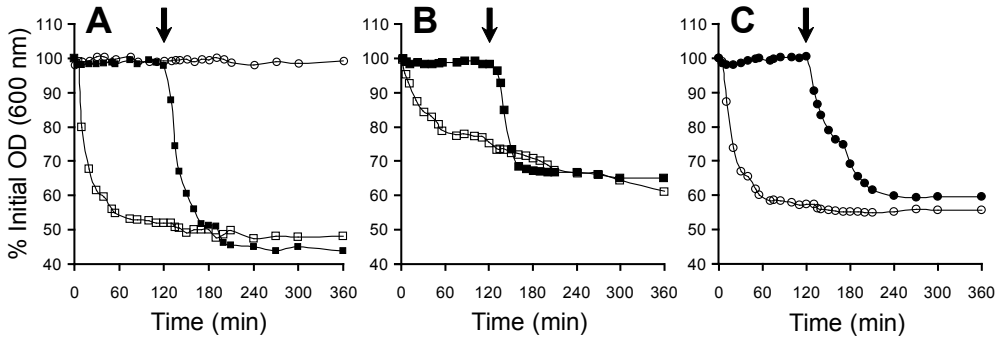

Fig. 1: Impact of pH and sorbic acid on amino acid- and inosine-induced germination of spores derived from *B. cereus* ATCC 14579 and its Δ*gerR* mutant. Heat-activated spores of *B. cereus* ATCC 14579 (squares) (A and B) and its Δ*gerR* mutant (circles) (A and C) were incubated at pH 5.5 (open symbols) or pH 5.5 plus 3 mM HSA (closed symbols). At time zero, the following germinants were used to induce germination: L-alanine (1 mM) (A), L-glutamine (1 mM) (B), and inosine (1 mM) (C). The vertical axis expresses the percentage of OD600 at the indicated time points, relative to that determined at time zero. The drop in optical density signifies germination. After 2 h of incubation, the pH in sorbic acid-stressed spore cultures was raised to pH 7.1 by addition of 2MNaOH (indicated with the black arrow). Graphs show the results from triplicate experiments.

blocks germination in *B. cereus* spores via the receptors GerR, GerG, GerQ, and GerI and does not display a receptor-specific effect, as was previously reported by Cortezzo for *Bacillus subtilis* spores (4). In the latter study, HSA arrested germination only when triggered by L-alanine in *B. subtilis*, but not when triggered by a mixture of L-asparagine, D-glucose, D-fructose, and potassium ions (AGFK). Notably, the concentration of sorbic acid used in the latter study (5 mg/liter) was far below the range (250 to 2,000 mg/liter) that is applied by industry (14), and given that experiments were performed at pH 6, the amount of undissociated acid was in the micromolar range. Inhibition of germination by HSA was for all tested nutrients a reversible process; when the pH was raised, spore germination is activated, as reflected in the drop in optical density (OD) indicative of the transition from phase bright to phase dark. Thus, in an extension of the previously reported data (3), inhibition of nutrient-induced germination of *B. cereus* by HSA does not involve competitive inhibition of (specific) germinant receptors.

Although the exact mechanisms following nutrient binding are not fully understood, several subsequent events are triggered upon binding of the nutrient to the receptor. After being committed to germinate, cations are released from the spores, followed by the release of Ca^{2+} and dipicolinic acid (DPA), which subsequently activates the cortex lytic system (8), allowing for expansion of the spore core by the uptake of water. Ca-DPA release involves the presence of SpoVA proteins that have been suggested to act as functional components of a specific channel (17). In this scenario, sorbic acid may display its inhibiting effect either by (i) directly blocking the Ca-DPA channel, (ii) inactivating the cortex lytic system, or (iii) preventing the signal transduction between nutrient-activated receptor(s) and the Ca-DPA channel.

To assess whether sorbic acid inhibits germination by directly blocking the Ca-DPA channel or by inactivating the cortex lytic system, dormant spores were exposed to either hydrostatic pressure (HP) or to exogenous Ca-DPA in the presence or absence of HSA. Ca-DPA is known

PhD Thesis Clint van Melis_corrected1.indd 61 4-12-2013 10:55:51

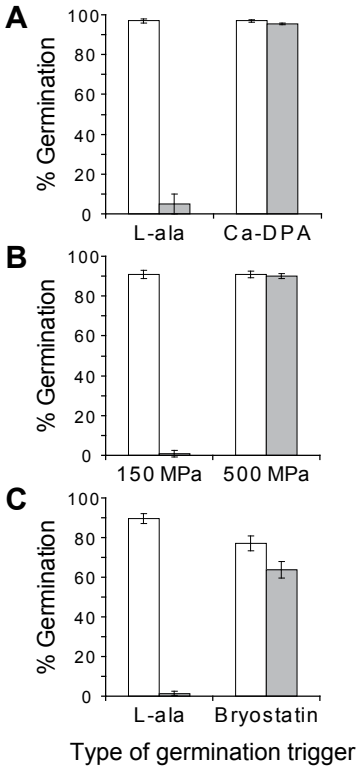

Fig. 2: Impact of sorbic acid on *B. cereus* ATCC 14579 spore germination induced by chemical and physical triggers. Heat-activated spores of *B. cereus* ATCC 14579 were incubated in MES buffer at pH 5.5 with 3 mM HSA (gray bars) or without HSA (white bars). The following germination triggers were used: a 1:1 chelate of 50 mM calcium and dipicolonic acid (Ca-DPA) (A), high hydrostatic pressure at either 150 or 500 MPa (B), and 10 µM bryostatin (C). The germination percentages were determined by comparing the number of germinated spores with the total number of spores present in each sample. The percentage bars show the results from duplicate experiments for high hydrostatic pressure and triplicate experiments for Ca-DPA and bryostatin, respectively.

to trigger spore cortex degradation by directly activating cortex lytic enzyme Cw1J (9, 10). Exposure of dormant spores to relatively high pressure (500 to 600 MPa) can induce germination either by the activation of channels in the inner membrane or by creating pores, with both possibilities resulting in the release of Ca-DPA (2). The triggering of germination by 100 to 200 MPa is assumed to involve activation of the nutrient receptors, even in the absence of nutrients, followed by activation of Ca-DPA channels (18, 19).

Heat-activated spores in MES buffer (pH 5.5) with or without 3 mM HSA were exposed to temperature-controlled pressure as previously described by Hornstra et al. (6) at 150 or 500 MPa or Ca-DPA at a final concentration of 50 mM. After exposure to HP (30 min) or to Ca-DPA (60 min), part of the samples was plated directly on brain heart infusion (BHI) plates (total number of spores), while a second part was heat treated for 10 min at 80°C before plating (only nongerminated spores survive) to determine the extent of germination. CFU were counted after overnight incubation at 30°C. Figure 2A and B show that spores were able to germinate when exposed to 50 mM Ca-DPA or 500 MPa, regardless of whether HSA was present or

PhD Thesis Clint van Melis_corrected1.indd 62 4-12-2013 10:55:52

not, thereby excluding an effect of sorbic acid on the cortex lytic system or Ca-DPA release. Only germination of *B. cereus* spores exposed to 150 MPa is affected by HSA, indicating that receptor-mediated signaling is inhibited. The fact that pressure-induced germination at 150 MPa without nutrients was still inhibited in the presence of sorbic acid supports the earlier assumption by Blocher and Busta (3) that a role for sorbic acid in competitive inhibition at the nutrient receptors is unlikely.

Recently, a novel mechanism for initiating spore germination was reported, in which germination is triggered by breakdown products of peptidoglycan (12). This route depends on the presence of the eukaryote-like Ser/Thr protein kinase PrkC, which is activated upon binding of cell wall peptidoglycan fragments or muropeptides. This newly identified germination pathway appears to act independently from the germinant receptor pathway. To test whether the PrkC pathway is sensitive to sorbic acid, both HSA- and non-HSA-exposed spores were induced to germinate by the addition of the cyclic macrolide bryostatin, a potent activator of the kinase-induced germination pathway (12). Heat-activated spores were incubated in MES buffer at pH 5.5 without or with HSA while being exposed to 10 μM bryostatin. After incubation for 120 min, an aliquot of the sample was plated directly on BHI plates, while a second aliquot was heat treated for 10 min at 80°C prior to plating (30°C overnight) to determine the proportion of (germinated) spores. Both control and HSA-exposed spores displayed a high percentage of germination when triggered with 10 μM bryostatin (Fig. 2C). The observed efficiency in the control (non-HSAexposed spores) is comparable to that of bryostatin-induced germination of *B. subtilis* spores (12). *B. cereus* spore germination in the presence of 3 mM HSA appeared to be only slightly reduced compared to that in non-HSA-exposed spores, which shows that activation and signaling of germination via the membrane-bound PrkC kinase is not prevented by HSA.

The results of this study allow us to draw several important conclusions about the action of HSA on spore germination. We demonstrated that HSA does not target specific germinant receptors, and in agreement with that finding, a role for sorbic acid in competitive inhibition of nutrients can thus be excluded. This is also supported by the fact that inhibition is instantaneously released when pH is upshifted. Additionally, the results show that inhibition of germination by sorbic acid can be bypassed by triggering later events of the germination pathway or by activating Ger protein receptor-independent germination pathways, suggesting that sorbic acid specifically interferes in the signaling between Ger receptors and putative ion/Ca-DPA channels. Insights provided by the present study may contribute to more efficient application of sorbic acid in food preservation, especially in those cases that involve mild thermal treatments. Here, it may be advisable to aim at intermediate HSA concentrations that prevent outgrowth, but do not completely inhibit germination, thereby maximizing spore inactivation at the thermal treatment step. Additionally, the data illustrate the importance of pH control in food preservation since small pH upshifts strongly affect HSA concentrations, which could lead to outgrowth opportunities for spores.

The mode of action of sorbic acid remains to be elucidated; however, two possible effects can be envisioned: (i) undissociated sorbic acid enters the spore and dissociates because of the higher

PhD Thesis Clint van Melis_corrected1.indd 63 4-12-2013 10:55:52

core pH (11), acidifying the spore's core and interfering with signaling; or (ii) the highly lipophilic undissociated sorbic acid accumulates in the core membrane and in this way interferes with signaling. The low mobility and reduced availability of water in the spore's core (15), the effects described for other nonacid lipophilic compounds (4), and the fact that a high concentration (15 mM) of undissociated acetic acid (pKa of 4.76, similar to that of sorbic acid, i.e., pKa 4.75) does not inhibit germination (although outgrowth is blocked) (1), combined with the immediate germination after pH upshift, suggest that the second mode of action is the most likely.

PhD Thesis Clint van Melis_corrected1.indd 64 4-12-2013 10:55:52

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PhD Thesis Clint van Melis_corrected1.indd 66 4-12-2013 10:55:52

Chapter 4

Germination inhibition of *Bacillus cereus* spores: impact of the lipophilic character of inhibiting compounds

C.C.J. van Melis, C. Bravo Almeida, R. Kort, M.N. Nierop Groot, T. Abee

Published in International Journal of Food Microbiology (2012), vol. 160 (2):124 – 130

PhD Thesis Clint van Melis_corrected1.indd 67 4-12-2013 10:55:52

In this study, the impact of a range of organic acids and structurally similar alcohols with three to six carbon backbones and increasing lipophilic character, were tested on the germination behavior of *B. cereus* ATCC 14579 spores. This approach allowed substantiating whether the effectivity of the various compounds was largely dictated by membrane interference or a classic weak acid acidification effect. The octanol–water partition coefficient (log Poct/water) ranges from 0.25/0.33 to 2.03/1.96 for propanol/undissociated propionic acid and hexanol/undissociated hexanoic acid, respectively. Performance of germination assays at neutral (pH7) and acidic conditions (pH5.5) allowed for a comparative analysis of the action of dissociated versus undissociated acids, and the presumed pH-independent effect of the corresponding alcohols. Germination assays, based on both continuously measured optical density and time-based plating experiments, and microscopic observations demonstrated the correlation between the lipophilic character of the selected compounds and their inhibiting effect on spore germination. Real-time fluorescence based assays showed that membrane integrity in dormant spores was maintained in the presence of the tested inhibitors. Lowering the critical concentration of inhibitors by a one-step washing procedure resulted in the onset of nutrient-induced germination, indicating the reversible nature of the inhibition process. Furthermore, blocking of nutrient-induced germination in the presence of inhibitory concentrations of selected lipophilic acids and corresponding alcohols was by-passed upon addition of Ca-dipicolinic acid, pointing to loss of signaling capacity in germinant receptor-mediated germination activity. These findings show that lipophilicity is an important determinant for the ability of the selected acids and corresponding alcohols to accumulate in the spore inner membrane and their ability to act as a germination-inhibitor.

Introduction

Weak organic acids are widely used by the food industry as effective preservatives in a variety of foods and beverages. These types of compounds find application for the control of a wide range of spoilage and pathogenic microorganisms, including control of germination and outgrowth of spores (van Melis et al., 2011a; Russell and Gould, 2003). Understanding the mode of action of weak acids on microbes is a prerequisite for the design of new and/or effective preservation strategies. Although quite a number of studies have appeared on the mode of action on vegetative cells (Smigic et al., 2010; Booth and Stratford, 2003; Eklund, 1983; Hirshfield et al., 2003; Krebs et al., 1983; Salmond et al., 1984; Stratford and Eklund, 2003), their impact on spore germination and subsequent outgrowth has not been sufficiently studied.

Despite the difference in chemical structure, all weak organic acids have in common that their inhibitory effect increases with decreasing pH. In the classical concept for the mode of action of weak organic acids on microbial cells, the protonated form of the molecule freely diffuses across the membrane lipid bilayer and dissociates inside the cell when it encounters the near neutral internal pH, resulting in concomitant acidification of the cellular cytoplasm (Bogaert and Naidu, 2000; Marshall et al., 2000; Russell and Gould, 2003; Setlow and Setlow, 1980).

PhD Thesis Clint van Melis_corrected1.indd 68 4-12-2013 10:55:52

Besides the impact on internal pH homeostasis, weak organic acids can also display an effect on integrity of the lipid membrane by accumulation in the inner membrane (Chu et al., 2009) and thereby interfere with its functioning (Stratford and Anslow, 1998; Brul et al., 2002). This effect is largely influenced by the lipophilic character of the compound, with more lipophilic compounds having a stronger tendency to accumulate in the lipid membrane (Stratford and Eklund, 2003).

We previously reported that the lipophilic organic acid sorbate inhibits *B. cereus* spore germination effectively (van Melis et al., 2011a, 2011b). Spore germination at pH 5.5 was completely blocked in the presence of 3 mM undissociated sorbic acid, whereas less lipophilic acetic acid prevented outgrowth but not germination at 15 mM of its undissociated form, even though both acids have similar pKa values. The relatively high pH of 6.2–6.4 in the core of dormant *B. cereus* spores (Setlow and Setlow, 1980) and the availability of free water in the spore core (Sunde et al., 2009) suggests that deprotonation of weak acids inside the spore core may be possible. However, the fact that sorbic acid releases fewer protons at its inhibitory concentration as compared to acetic acid and the fact that other, non-acidic, lipophilic compounds can also inhibit spore germination (Cortezzo et al., 2004; Yasuda-Yasaki et al., 1978) favor a model in which the inhibiting character of these compounds is more based on interference with germination-signaling by accumulation in the core membrane than by acidification of the spore's core (van Melis et al., 2011b). Germination inhibition of *B. subtilis* spores by nonacidic lipophilic compounds was suggested to specifically target germination via the L-alanine germinant receptor (Cortezzo et al., 2004; Yasuda-Yasaki et al., 1978). In contrast, sorbic acid completely blocked *B. cereus* spore germination in nutrient rich BHI broth and also when several of the seven Ger-receptors present in *B. cereus* spores (Hornstra et al., 2006) were triggered individually in a buffer-solution, pointing to a mode of action that is less germinant receptor-specific (van Melis et al., 2011b). Observations that germination blockage by sorbic acid and other selected, non-acidic, lipophilic compounds is reversible (Cortezzo et al., 2004; Yasuda-Yasaki et al., 1978) and can be relieved by germination induction in later stages of the germination pathway (van Melis et al., 2011b) support the hypothesis that lipophilic compounds affect germination by accumulation into the spore's inner membrane. This accumulation could in turn affect the inter-dependent clusters of proteins located therein, that are involved in nutrient induced spore germination (Griffiths et al., 2011; Vepachedu and Setlow, 2007).

To substantiate that the effectivity of lipophilic acid compounds to prevent spore germination is dictated by accumulation into the inner membrane, we analyzed the germination inhibiting effects of a range of organic acids and alcohols, with varying degrees of lipophilicity. Acids and corresponding alcohols with carbon back bones ranging from C3 to C6 were selected, with logPoct values ranging from 0.25 to 2.03. This parameter has been shown to be an excellent indicator for their efficiency of accumulation into artificial membranes (Jain et al., 1978). The lipophilic character of weak acids is, in contrast to alcohols, strongly dependent on the pH, that is the protonated acid is much more liphophilic than the dissociated acid. Membrane interference and protonation/deprotonation effects have been described as important for the mode of action of weak acids for growth inhibition of vegetative cells (Salmond et al., 1984; Booth and Stratford, 2003; Stratford and Eklund, 2003). The present study investigates the importance of

PhD Thesis Clint van Melis_corrected1.indd 69 4-12-2013 10:55:52

acidity and lipophilicity for the inhibition of spore germination by assessment of spore behavior upon exposure to organic acids and alcohols at both a neutral pH (pH 7) and a mildly acidic pH (pH 5.5). A novel fluorescence-based viability assay (Kort et al., 2010), known to be sensitive to the state of the cell membrane (Nocker et al., 2011; Kort et al., 2010), was used in this study to assess membrane integrity in dormant spores in the presence of the tested inhibitors.

Materials and Methods

Strain and culture conditions

Bacillus cereus strain ATCC 14579 was obtained from the American Type Culture Collection (ATCC) and stored in Brain Heart Infusion (BHI) broth supplemented with 50% glycerol at−80 °C. Unless stated otherwise, *B. cereus* was cultivated at 30 °C with aeration at 200 rpm (Innova 4335 Incubator Shaker, New Brunswick Scientific, United States). Spores of *B. cereus* were harvested from cultures grown in defined, minimal sporulation medium described previously (de Vries et al., 2004). Spores were prepared as described previously (van Melis et al., 2011a) with the following modification: the Tween 80 concentrations were reduced from 0.1% to 0% in a timespan of five days. Pure spore crops devoid of vegetative cells and debris were stored in phosphate-buffered saline (PBS, pH 7) at 4 °C for not more than 8 weeks until use.

Spore germination assay

Spore germination was assessed under either unstressed conditions (pH 7) or mildly acid-stressed conditions (pH5.5) in Brain Heart Infusion broth (BHI). The BHI was supplemented with a range of organic acid- or alcohol-concentrations with various lipophilic properties at the aforementioned pH values. Spore germination was followed by the transition of phase bright spores turning phase dark, which is observed as a drop in optical density in a spectrophotometer (Bioscreen C, Oy Growth Curves Ab Lt., Finland). A drop in OD600nm to 45% of starting OD corresponds with >99% germination. The spore density was adjusted in all experiments to an OD600nm of 0.8–0.85 to ensure that similar numbers of spores were used in all experiments (final concentrations of ~$2 \bullet 10^8$ spores/ml). Prior to initiation of germination, spores were pelleted by centrifugation at maximum speed for 30 s (Eppendorf tabletop centrifuge, Germany), washed and recovered in PBS. The PBS was prepared with a pH similar to that used in the test condition to prevent pH up-shifts affecting the concentration of undissociated acid. Aliquots of 20 µl of the dormant-spore crop was added to multiple wells of a 100 wells 'honeycomb' microtiter plate, kept on ice. This was followed by the addition of 180 µl of 1.1× concentrated buffered BHI (100 mM MES buffer, pH7 or 5.5, depending on the test condition), supplemented with the desired organic acid or alcohol. Within 2 min after addition of the acid or alcohol, the microtiter plate was loaded into the pre-heated Bioscreen spectrophotometer. The plate was incubated at 30 °C for 120 min and optical density was measured every 3 min. For each condition 3 independent biological replicates (from independently prepared spore suspensions) were used. Each replicate was repeated 3 times (technical replicates), resulting in 9 data points per condition.

Replicates were very repeatable, both within each plate and between the biological replicates, resulting in very small standard deviations (standard deviation was typically around 1.4% and maximum deviation was 3.6%). Microscopic examination of spores prior to and after experi-

PhD Thesis Clint van Melis_corrected1.indd 70 4-12-2013 10:55:52

ments was performed routinely to verify OD-based observations. Additionally, aliquots from the test wells (one representative per biological replicate) were plated in duplicate onto BHI plates to determine Total Viable Count and Spore Counts (10 min 80 °C prior to plating). Germination percentages were calculated by subtracting the SC CFUs from those of the TVC, and this number was subsequently divided by the CFU counts from the TVC.

Real-time fluorescence assay (for assessment of spore membrane integrity)
Membrane integrity in the presence of germination-inhibiting concentrations of the lipophilic compounds was determined by measuring the fluorescence emission in a so-called real-time viability assay (Kort et al., 2010). In short, this assay is based on phototautomerism of a weak acid probe after entering the spore core. The transition from a low pH outside to a neutral pH inside the core induces the probe to dissociate into a fluorescent phototautomeric anion. Since the level of fluorescence emission is proportional to the internal pH of cells and spores, membrane damaged spores with more acidic core pH can be distinguished from spores with intact membranes and a neutral core-pH. Heat-treated spores (20 min at 100 °C) were used as controls. Aliquots of 500 µl of spore solution (~$2 \bullet 10^8$ spores/ml) were washed by centrifugation twice in 10 mM PBS of either pH7 or pH5.5, depending on the incubation conditions. After washing the pellets were taken up in 500 µl of 10 mM PBS, containing the desired concentration of lipophilic compound, and incubated for 15 min at 30 °C to allow the compounds to either accumulate into the inner membrane or into the spore core. Subsequently, 100 µl aliquots were transferred into the wells of a 96-well microtiter plate (Greiner, Flat bottom, UV-star) and inserted into a Tecan Infinite F500 microplate reader (Tecan Group Ltd, Switzerland). The phototautomeric probe salicylic acid proved to be themost efficient probe to be used in the real-time viability assay in combinationwith *B. cereus* ATCC 14579 spores (data not shown). Identical volumes of this probe were injected in each sample, right before initiation of the experiments. Probes were typically dissolved in 200 mM phosphate buffer pH 1.4 with a probe concentration of 2 mM. The plate reader settings for measurement were as described by Kort et al. (2010) with the following exceptions: manual gain, 55; number of flashes, minimal 20; probe injection after 10 cycles; shaking 3 s, orbital 2 mm, and wait time 3 s. Fluorescence was measured continuously during 8 h with excitation at 280 nm and emission at 400 nm. All compounds were tested in quintuple reactions and the experiment was performed with a biological duplicate.

Spore germination induced by exposure to Ca-DPA
Dormant *B. cereus* spores were incubated for 15 min in MES-buffer (pH 5.5) in the absence and presence of sorbic acid (3 mM), propanoic acid/propanol (60 mM), or hexanoic acid/hexanol (2.5 mM). The buffer was supplementedwith 1 mM L-alanine as a germinant. After exposure to 50 mM of Ca–DPA (30 min), part of the samples was plated directly on BHI plates (total viable count),while a second part was heat-treated for 10 min at 80 °C before plating (spore count) to determine spore counts, and the extent of germination was calculated by subtracting the percentage of heat-resistant spores fromthe total number of colony forming units, divided by the total viable counts. Colony forming units were counted after overnight incubation at 30 °C. For each condition three replicates were tested (technical replicates) and the experiment was performed twice (biological duplicates).

PhD Thesis Clint van Melis_corrected1.indd 71 4-12-2013 10:55:52

Results

The impact of lipophilic compounds on *B. cereus* spore germination was tested by following the germination of spores in BHI, supplemented with a range of concentrations of alcohols and organic acids with different chain-lengths (Table 1). Germination was followed in optical density-based germination assays at both a neutral pH of 7 and mild acidic pH of 5.5 (Fig. 1).

To substantiate the impact of dissociated versus undissociated acid on spore germination, similar amounts of acids were added at both neutral (pH 7) and acidic (pH 5.5) conditions. All weak acid solutions were prepared to obtain 3 mM undissociated acid at pH 5.5, according to methods described previously for sorbic acid (van Melis et al., 2011b). The concentration of undissociated acids was significantly lower at pH 7 as the equilibrium is shifted towards the dissociated form of the acid, resulting in concentrations of undissociated acids ranging from 0.44 mM for the least lipophilic to 0.02 mM for the most lipophilic acids. Fig. 1 clearly shows the impact of pH on the inhibiting properties of the tested acids (Panels A and B), as inhibition only occurs at the mild pH-stressed conditions of panel B. Complete inhibition of germination was reached for each of the tested acids when the concentration of undissociated acid was further increased (typically between 5 and 100 mM for the most and least lipophilic compounds, respectively; data not shown). Exposure to 3 mM undissociated acid for each of the individual organic acids at pH5.5 clearly shows that inhibition of germination increased with the chain-length of each of the tested compounds (Fig. 1, panel B).

The presence of 3 mM of the individual alcohols did inhibit spore germination, but the inhibition was not pH-dependent (Fig. 1, panels C and D) albeit that the germination rate at pH5.5 is slightly reduced with all compounds as compared to pH 7. In contrast to their acid counterparts, there is no significant difference between the inhibitory properties of the C3 (propanol) and C4 (butanol) compounds when added at 3 mM; differences in inhibitory properties between these compounds only become visible at concentrations above 15 mM (Fig. 2).

Table 1: Overview of lipophilic acids and alcohols used in this study.

Overview of lipophilic acids and alcohols used in this study

Compound	Carbon chain length	logP_{oct}[a]	pK_a	Concentration with >95% inhibition (mM, pH 5.5)[b]	Concentration of released protons (mM)[c]
Propionic acid	C3	0.33	4.87	58.5 ± 4.7	1.677
Butyric acid	C4	0.79	4.82	61.1 ± 3.6	1.566
Valeric acid	C5	1.29	4.82	20.2 ± 1.1	0.578
Sorbic acid	C6	1.33	4.76	2.9 ± 0.3	0.065
Hexanoic acid	C6	1.92	4.88	2.5 ± 0.2	0.073
Propanol	C3	0.25	-	> 100	-
Butanol	C4	0.61	-	>100	-
Pentanol	C5	1.19	-	23.3 ± 4.1	-
Hexanol	C6	2.03	-	2.5 ± 0.4	-

a logP_{oct} values were calculated with Episuite 4.1.
b In case of the acids, the concentration of undissociated acid was used.
c Concentration of released protons, assuming an intrasporal pH of 6.4 in *B. cereus* spores (Setlow and Setlow, 1980), calculated for the situation that all acid enters the spore core.

PhD Thesis Clint van Melis_corrected1.indd 72 4-12-2013 10:55:53

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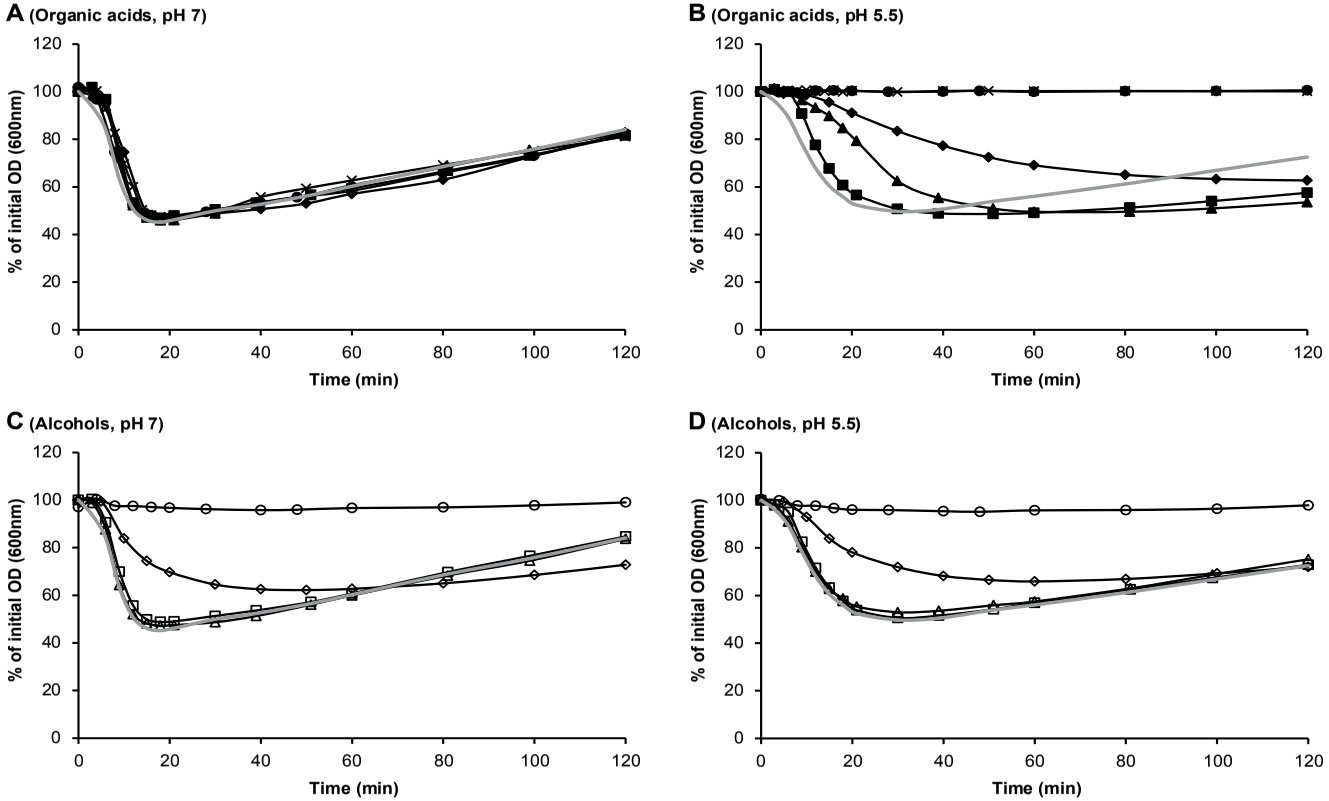

Fig. 1: Germination and outgrowth of spores in the presence of organic acids and alcohols with increasing lipophilic character, expressed by the changes in optical density. Spores were incubated at 30 °C in the presence of one of the following organic acids in panels A and B (propionic acid (squares), butyric acid,(triangles), valeric acid (diamonds), hexanoic acid (circles), or sorbic acid (x) or alcohols in panels C and D (propanol (open squares), butanol (open triangles), pentanol (open diamonds), or hexanol(open circles). Spores were incubated at either pH7 (panels A and C) or pH5.5 (panels B and D). Controls of spores germinating without inhibitors are shown in the grey lines without markers for pH 7 (panels A and C) and pH 5.5 (panels B and D). For the organic acids, overall concentrations were calculated to obtain 3 mM undissociated acid at pH5.5. Identical overall concentrations were used for each acid at pH7. Incubations in the presence of alcohols were performed at concentrations of 3 mM. The y-axis shows the change in optical density (OD) relative to the OD at initiation of germination. Graphs show the average results of triplicate experiments.

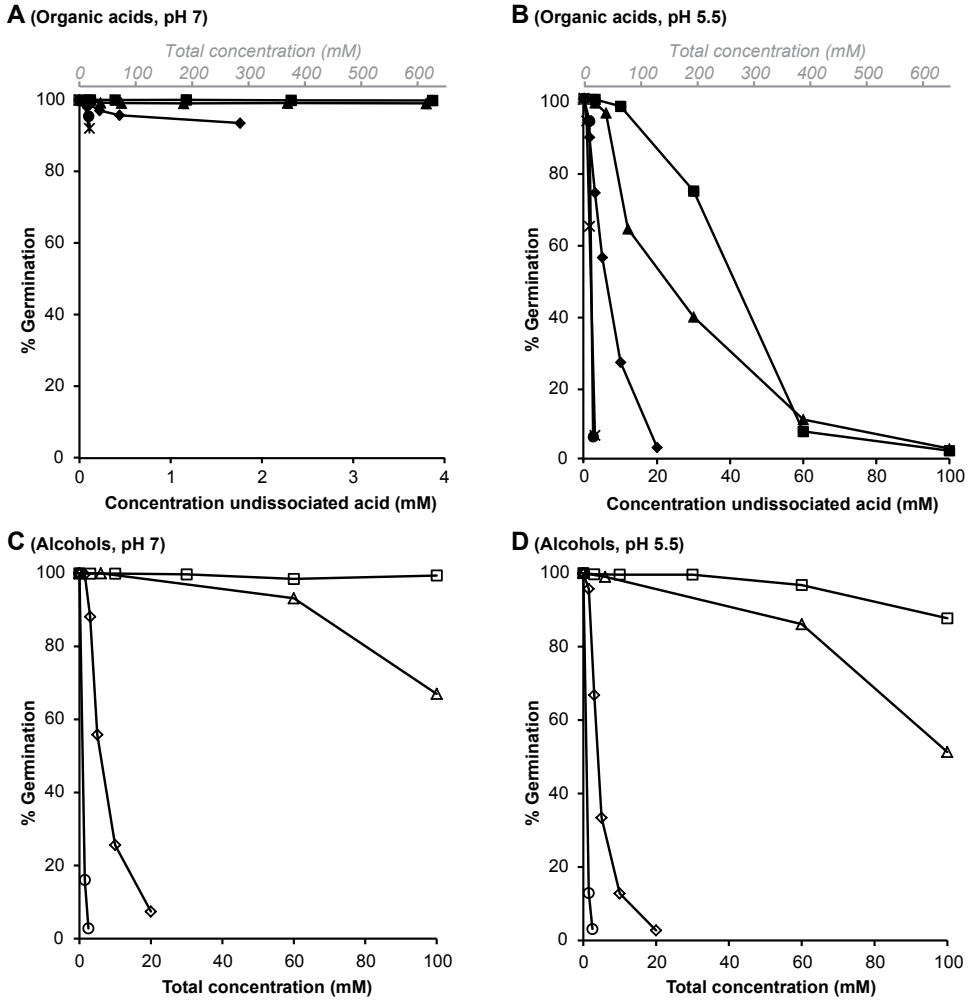

Fig. 2: Germination inhibition of *B. cereus* spores in the presence of concentration ranges of selected acids and alcohols, after 60 min of incubation at 30 °C. Spores were incubated in the presence of one of the following organic acids: propionic acid (squares) , butyric acid (triangles), valeric acid (diamonds), hexanoic acid (circles), or sorbic acid (x) or alcohols: propanol (open squares), butanol (open triangles), pentanol (open diamonds), or hexanol (open circles). Spores were incubated at either pH7 (panels A and C) or pH5.5 (panels B and D). For the alcohols and organic acids, identical overall concentrations were used at pH7 and pH5.5, and germination data in the presence of acids (panel A and B), plotted as a function of the respective concentrations of undissociated acid. Graphs show the average results of triplicate experiments.

The calculated germination percentages in the presence of concentration ranges of lipophilic organic acids and alcohols were linked to the tested concentrations of each of the compounds (Fig. 2). For the selected alcohols, the concentrations used were similar to that of the corresponding undissociated acid (with the same carbon chain length), that gave >95% inhibition of germination at pH 5.5. In the experiments at pH 7 (Fig. 2, panel A) similar overall concentrations of the different acids were used as at pH5.5. Based on pKa values, the resultant concentrations of undissociated acids were 15–20 times lower at pH 7 compared to that at pH 5.5. These low concentrations of undissociated acid did not significantly inhibit germination.

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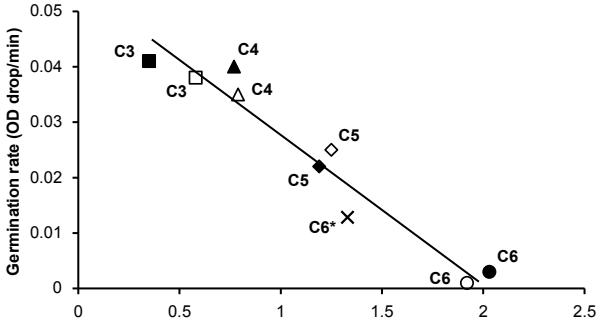

Fig. 3: Scatterplot of *B. cereus* spore germination rate versus the partition coefficient (logPoct) of the tested compounds. The germination rates represent the slope of the OD-changes that resulted from germination at pH5.5, in the presence of 1.5 mM undissociated acid (open symbols) or alcohol (closed symbols) of one of the following compounds; propionic acid/propanol (C3, squares), butyric acid/butanol (C4, triangles), valeric acid/pentanol (C5, diamonds), hexanoic acid/hexanol (C6, circles), or sorbic acid (C6*, x). The displayed trend line has a coefficient of determination (R^2) of 0.937. The calculations for germination rate are based on duplicate biological experiments.

The exposure at pH 5.5 (Fig. 2, panel B) shows that germination can be efficiently blocked with low concentrations of undissociated sorbic acid (3 mM) and hexanoic acid (2.5 mM), medium concentrations of undissociated valeric acid (20 mM) and high concentrations of undissociated butyric acid and propionic acid (60 mM).

In contrast to the acids, the impact of the alcohols was not dictated by the pH (Fig. 2, panels C and D). Only small differences in germination efficiency could be observed between spores incubated with the alcohols at pH 7 and pH 5.5, but control data sets indicated that these differences could be attributed to a pH effect only (data not shown). Hexanol and pentanol inhibit germination at concentrations that are very similar to those of the corresponding acids with the same chain-length, in their undissociated forms. In contrast, this is not the case for butanol and propanol. These short-chain alcohols allow high levels of germination at concentrations where similar concentrations of undissociated propionic acid and butyric acid completely blocked germination. Notably, total amounts of propionic and butyric acid are very high under these conditions, reaching concentrations of 376 and 347 mM, respectively.

Germination rates in the presence of low concentrations of the tested compounds were calculated based on the changes in optical density. These germination rates were then correlated with the partition coefficient of each of the used compounds. The maximum concentration of undissociated acid where germination in the presence of all compounds could still occur (1.5 mM) was chosen to show this correlation (Fig. 3). This figure shows that there is a strong correlation (R^2=0.937) between the reduction in germination rate and the lipophilic character of a given compound, with the more lipophilic compounds inhibiting germination rate more efficiently.

Accumulation of lipophilic compounds into the inner membrane may affect membrane integrity, thereby allowing acidification via the influx of protons. To test this possibility, we performed a real-time fluorescence assay that was previously designed as a fluorescence-based method to assess viability of cells and spores (real-time viability assay; Kort et al., 2010). This

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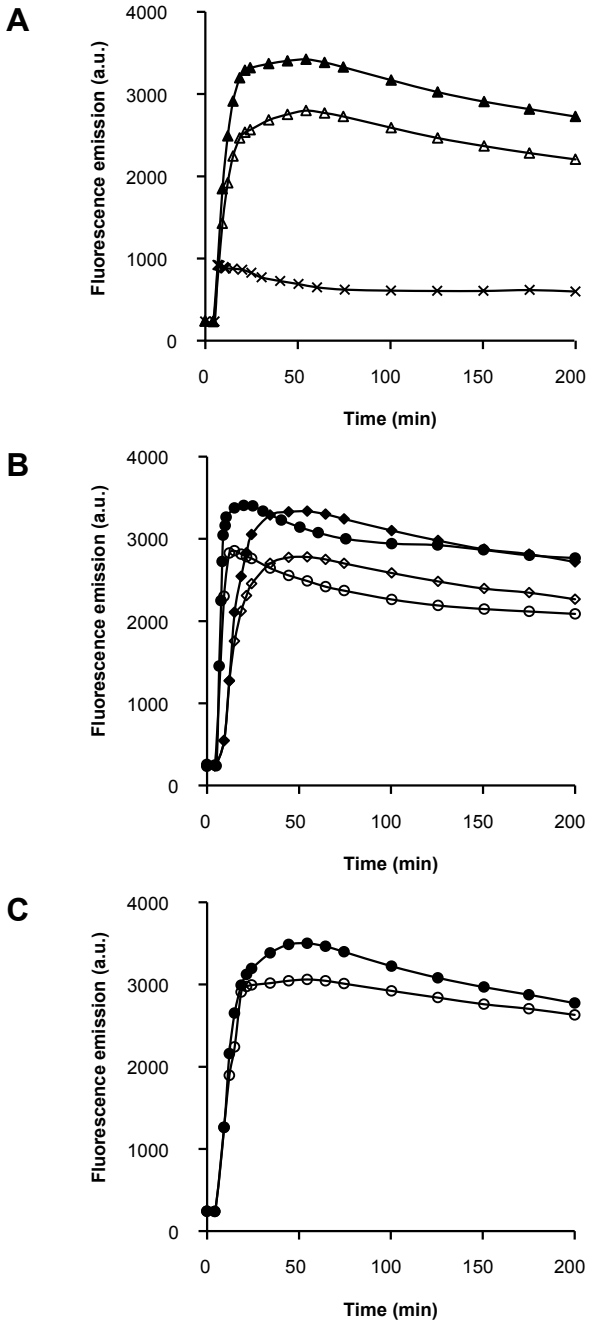

Fig. 4: Real-time viability assay kinetics obtained under germination-inhibiting conditions. Spores were incubated at 30 °C, either in absence of lipophilic compounds (panel A, triangles), or in the presence of lipophilic acid (panel B) using sorbic acid (3 mM and 0.11 mM undissociated acid at pH 5.5 and pH 7, respectively, diamonds) or hexanoic acid (2.5 mM and 0.10 mM undissociated acid at pH 5.5 and 7, respectively, circles). In panel C, hexanol (2.5 mM, circles) was added. Incubations were performed at either pH7 (closed symbols) or pH5.5 (open symbols). Heat damaged spores (20 min 100 °C, crosses, panel A) were used as a control. Graphs show the average results of duplicate experiments.

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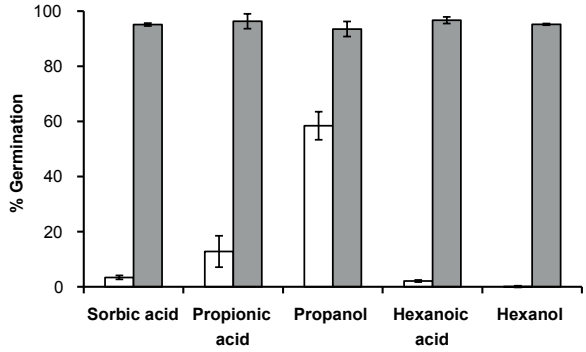

Fig. 5: Germination of *B. cereus* ATCC 14579 spores in the presence of inhibiting concentrations of alcohols and organic acids. Dormant spores were incubated at 30 °C and in the presence of sorbic acid (3 mM), propionic acid (60 mM), propanol (60 mM), hexanoic acid (2.5 mM) and hexanol (2.5 mM), where the concentrations for the acids are the undissociated concentrations. Germination was triggered by the addition of Ca–DPA to reach final concentrations of 50 mM (dark bars), while the absence of Ca–DPA was used as a control (white bars). Bars represent results of triplicate experiments and the error bars represent the standard deviation within these results.

assay is based on the principle that a neutral weak acid probe dissociates into a fluorescent phototautomeric anion in intact cells with neutral cytosol. The dissociation of this probe depends on the presence of a ΔpH and an intact inner membrane. An increase in fluorescence in this assay can only occur when the integrity of the inner membrane is not compromised and the internal pH of spores is in the near-neutral range. Fluorescence kinetics of spores were measured in the presence of hexanol, hexanoic acid and sorbic acid (Fig. 4) with non-stressed and heat-treated spores serving as controls. In the assay, dormant spores that were boiled for 20 min (crosses) virtually no increase in fluorescence levels compared to spores incubated with the other tested compounds, showing a clear baseline for membrane damaged spores. Kinetics for spores, either in the absence (Fig. 4, panel A) or presence of alcohols or organic acids (Fig. 4, panels B and C, respectively) show that the intensity of the transient fluorescent signal is slightly reduced when spores were incubated at pH5.5. Similar fluorescence kinetics were observed for spores that were incubated in the presence of propanol/propionic acid, butanol/butyric acid and pentanol/valeric acid (data not shown). Spores that were incubated in the presence of all (lipophilic) compounds tested in this study display relatively high transient fluorescence levels in the real-time viability assay, comparable to those obtained in the absence of these compounds and at the same pH, suggesting that membrane integrity is not affected.

Previous work showed that the germination-inhibition by 3 mM undissociated sorbic acid could be circumvented by the addition of Ca–DPA (van Melis et al., 2011b). A similar experiment was performed in the presence of the C3 and C6 compounds used in this study to assess whether the observations for sorbic acid also apply to these compounds. Fig. 5 shows that spores arrested in germination by the addition of propionic acid, hexanoic acid and hexanol could resume germination when Ca–DPA was added, showing that the inhibition by these compounds could be circumvented, similar to what was observed for high concentrations of undissociated sorbic acid.

PhD Thesis Clint van Melis_corrected1.indd 77 4-12-2013 10:55:54

Discussion

We have performed a comparative analysis of the effect of structurally similar organic acids and alcohols on the germination behaviour of *B. cereus* spores to substantiate whether a role as membrane-active compound or acidifying compound dominates. The compounds with the longest chain-length displayed a stronger im pact on germination than the short chain compound. The inhibition properties of the organic acids are pH dependent, since both their membrane-passing and lipophilic properties are determined by their undissociated form, whereas the impact of the alcohols is independent of pH. The most lipophilic compounds with 6 carbons, including the alcohol analogues, blocked germination in concentrations (2 to 3 mM) similar to those previously observed for sorbic acid (van Melis et al., 2011a). The fact that blockage of germination could be by-passed by the addition of Ca–DPA, as was previously observed for sorbic acid (van Melis et al., 2011b), suggests that these medium chain lipophilic compounds interfere with nutrient-mediated signal transduction as was demonstrated for sorbic acid (van Melis et al., 2011b).

To substantiate which feature of medium chain lipophilic acids dominates (acidifying effect on spore core or membrane-active compound) we exploited the fact that in contrast to acids, alcohols cannot dissociate and acidify the spore's core and inhibiting effects can only be caused by interference with the spore membrane. The fact that for the most lipophilic compounds (C5 and C6 chain lengths) no significant differences were apparent between the acid and alcohol analogues in their inhibiting properties, whereas for the least lipophilic compounds only the acid analogues affected spore germination, indicates that the inhibiting character of the tested compounds can be influenced by both their acidity and their lipophilic character, with contribution of the latter becoming more important with increasing chain-length.

We have demonstrated that the mode of action of medium chain acids was dominated by its relatively high lipophilicity which favors accumulation in the inner membrane of the spore. In this scenario, there are two hypotheses for its mechanism of inhibition:

1) The accumulation of alcohols into the spore's inner membrane could disrupt the phospholipid layers and affect membrane integrity, such as has been shown to be the case with ethanol in the cytoplasmic membrane of *E. coli* cells (Fried and Novick, 1973; Ingram and Buttke, 1984; Ingram and Vreeland, 1980) and similar effects have been suggested for medium-chain fatty acids such as valeric-, hexanoic- and sorbic acid (Stratford and Anslow, 1998; Stratford and Eklund, 2003) and long-chain fatty acids such as lauric acid and linoleic acid (Ababouch et al., 1994).

2) The accumulation of foreign compounds into the inner membrane will adjust the membrane's volume (Griepernau et al., 2007; Griepernau and Böckmann, 2008; Sikkema et al., 1994) and this could have an influence on protein-protein interactions in the inner membrane without affecting membrane integrity. Recently, Griffiths et al. (2011) described a model where proteins essential for nutrient-induced germination of spores are clustered in close proximity in so-called 'germinosomes,' consisting of interdependent clusters of each of the contributing proteins. Volume changes and saturation of the inner membrane by the accumulation of lipo-

PhD Thesis Clint van Melis_corrected1.indd 78 4-12-2013 10:55:54

philic compoundsmay have an impact on the protein–protein interactions between the clusters in these 'germinosomes.'

In the first hypothesis, where spore germination is inhibited by affecting membrane integrity, there should be a significant effect on viability of the outgrowing spore. Our observations that the inhibition by lipophilic compounds is both reversible and can be bypassed by inducing germination at post-germinant receptor stages suggest that the viability of affected spores is not reduced. Furthermore, the maximum fluorescence intensity (real-time viability assay; Kort et al., 2010) at pH 5.5 is comparable for conditions without and with lipophilic alcohol or acid, thereby discrediting the hypothesis of a compromised membrane integrity or membrane disruption.

With the above in mind, the second hypothesis, where the clusters of the 'germinosomes' are separated by changes in membrane volume and its saturation with lipophilic compounds appears more likely. Using *B. subtilis* spores, Griffiths et al. (2011) confirmed interactions between clusters consisting of one of the germinant receptors and GerD and also between GerD and SpoVA protein-clusters, which are expected to be involved in the release of dipicolinic acid (Vepachedu and Setlow, 2007). These proteins are relatively immobile during the first stages of spore germination (Cowan et al., 2004), and the less abundant germinant receptors need therefore to be localized in the close proximity of the more abundant GerD and SpoVA proteins for rapid and successful germination. If these interdependent clusters are separated by the accumulation of the lipophilic compounds, signal transference could very well be impaired, preventing the spores from germinating. Our observations that inhibition was reversible, when the concentrations of these compounds in the inner membrane were reduced, further reinforce this hypothesis.

Our work has demonstrated that the effectivity of alcohols and weak organic acids in preventing spore germination is largely based on their lipophilic character. This may provide leads for the screening of novel antimicrobials and development of more efficient combinations of (novel) food preservatives for general or specific applications, dependent on microbial target organism(s) and relevant characteristics of food products including acidity and fat content.

PhD Thesis Clint van Melis_corrected1.indd 79 4-12-2013 10:55:54

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PhD Thesis Clint van Melis_corrected1.indd 82 4-12-2013 10:55:54

Chapter 5

Impact of sorbic acid on germination and outgrowth heterogeneity of *Bacillus cereus* ATCC 14579 spores

H. M. W. den Besten, C. C. J. van Melis, J.-W. Sanders, M. N. Nierop Groot, T. Abee

Published in Applied and Environmental Microbiology (2012), vol. 78 (23):8477 – 8480

PhD Thesis Clint van Melis_corrected1.indd 83 4-12-2013 10:55:54

Population heterogeneity complicates the predictability of the outgrowth kinetics of individual spores. Flow cytometry sorting and monitoring of the germination and outgrowth of single dormant spores allowed the quantification of acid-induced spore population heterogeneity at pH 5.5 and in the presence of sorbic acid. This showed that germination efficiency was not a good predictor for heterogeneity in final outgrowth.

Bacillus cereus is a food-spoiling and food-poisoning spore-forming organism that can be found in a large variety of foods and food ingredients (10). It has the ability to survive in harsh environments because it can form endospores that are resistant to heat, dehydration, and other physical and/or chemical stresses, and these spores may germinate and grow when conditions become more favorable. Thermal preservation methods that ensure complete inactivation of highly stress-resistant spores will negatively affect food quality, and the current trend toward milder preservation methods advocates application of mild food preservation factors, including mild heat treatments combined with addition of weak organic acids (11), to delay or inhibit germination and outgrowth of spores and cells.

We previously investigated the impact of sorbic acid (SA) on germination and outgrowth of *B. cereus* spores for a population as a whole (18), but heterogeneity in outgrowth between individual spores in the population was not quantified and will affect the germination and outgrowth profile. Furthermore, it is well documented that heat treatments can trigger activation of spores and accelerate germination (7) and thereby can influence outgrowth dynamics and possibly also heterogeneity. To date, the impact of heat treatment on germination and outgrowth kinetics has been described at the population level (for example, see references 8 and 16), but heterogeneity at the single-cell level has not been studied in combination with mild (acidic) stress factors to control the germination and outgrowth of *B. cereus* spores. Therefore, we assessed the impact of SA on germination and outgrowth kinetics at both the population level and single-cell level for spores that were heat shocked (HS) prior to initiation of germination. For that purpose, *B. cereus* ATCC 14579 spores were harvested from cultures grown in defined, minimal sporulation medium (4) and prepared as described previously (18) with the following modification: the Tween 80 concentrations were reduced from 0.1% to 0% in five daily washing steps. Pure spore crops devoid of vegetative cells and debris were stored in phosphate-buffered saline (PBS; pH 7) at 4°C for at least 2 weeks and not more than 8 weeks until use. Spore germination was assessed under either unstressed conditions (brain heart infusion [BHI] buffered with 100mMPBS [bBHI]; pH 7) or under mild acid stress (bBHI at pH 5.5 or bBHI at pH 5.5 with supplementation with 0.75mM undissociated sorbic acid [HSA]). Spore germination was monitored by the transition of phase-bright spores turning phase dark, which is observed as a drop in optical density (OD) in a spectrophotometer (Versa-Max, Molecular Devices, United States) as described previously (5). Spore density was adjusted in all experiments to an optical density at 600 nm (OD600) of 0.8 to 0.9 to ensure that similar numbers of spores were used in all experiments (final concentration, ~$2 \bullet 10^8$ cfu/ml). Spores were either mildly heat shocked at 70°C for 10 min or not heat shocked by incubation at room temperature. After heat shock and

PhD Thesis Clint van Melis_corrected1.indd 84 4-12-2013 10:55:54

prior to initiation of germination, spores were washed in PBS of the appropriate pH (pH 7 for the spores that were incubated at pH 7 and pH 5.5 for the spores germinated at pH 5.5 without and with addition of 0.75 mM HSA). Twenty microliters of spore solution was transferred to 250µl wells in precooled microtiter plates, followed by addition of 180 µl of 1.1x concentrated bBHI at either pH 7 or pH 5.5 or at pH 5.5 with supplementation with 0.75mMHSA. The OD600 was measured every 120 s for 8 h in a spectrophotometer (VersaMax; Molecular Devices, United States) that was prewarmed to 30°C. For each condition, two independent biological replicates were tested. Each replicate was repeated in eight wells (technical replicates), resulting in 16 data points per condition.

The optical density measurements showed that lowering the pH to 5.5 increased the lag time to initiation of germination (Fig. 1). The lag time increased further in the presence of 0.75mMHSA at pH 5.5. The maximum drop in OD represented close to 100% germination at pH 7 and 5.5, as confirmed by plate counting of spores and cells (data not shown), whereas in the pres-

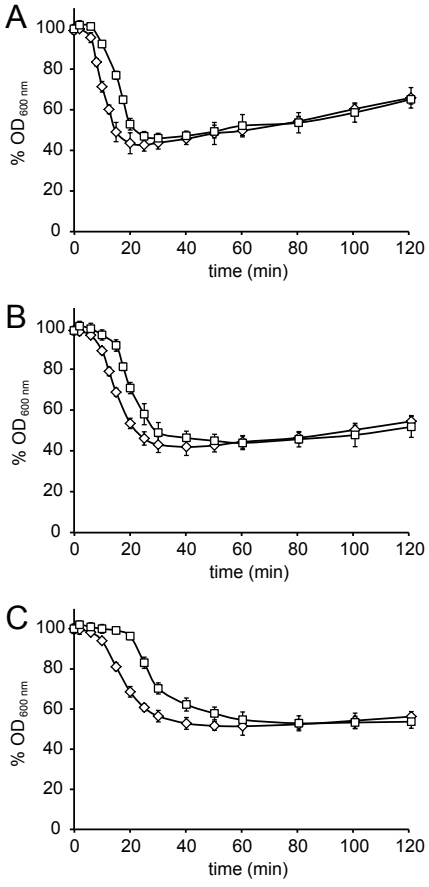

Fig. 1: Impact of heat treatment and subsequent acid stress exposure on germination and initial outgrowth kinetics. Prior to germination, dormant spores were heat-shocked for 10 min at 70°C (diamonds) or not heat-shocked (squares), after which germination and outgrowth was monitored at 30°C in buffered BHI at pH 7 (a), pH 5.5 (b), and pH 5.5 supplemented with 0.75 mM undissociated sorbic acid (c). Error bars represent standard deviations of the repetitions (n=16).

PhD Thesis Clint van Melis_corrected1.indd 85 4-12-2013 10:55:55

ence of 0.75 mM HSA, the germination efficiency was slightly lower and approximately 95%. Reduced germination efficiency by HSA may be caused by accumulation of sorbic acid, which is relatively hydrophobic, in the spore's inner membrane, and which may interfere with the signaling cascade in germinant-receptor-mediated germination (17). Heat shock reduced the time to reach a maximum OD drop at pH 7 and under both acidic conditions, underlining that the rather mild heat treatment applied in this study resulted in activation of spores and acceleration of germination. Acceleration of germination by heat treatment is a known phenomenon, and recently, it has been suggested that heat activation might increase the responsiveness of germination receptors and faster release of dipicolinic acid (DPA) (21). We now observed that this acceleration is still significant when combined with germination-delaying factors like the presence of sorbic acid.

Germination curves as shown in Fig. 1 represent the summation of behavior of individual spores within a population, and mild preservation stresses have been shown to induce heterogeneity in growth potential. Both low temperature and salt stress exposure have been reported to induce heterogeneity for exponentially growing *B. cereus* cells (2, 3), but data on stress-induced heterogeneity for dormant spores are not available. Heterogeneity for germination and outgrowth of spores complicates the predictability of spore behavior in response to preservation treatments like acidification. Single-cell performance analysis provides more accurate insight into population heterogeneity and is necessary to better understand and quantify the heterogeneity in germination and outgrowth potential in an acidic environment. Therefore, growth curves initiating from single dormant spores were obtained using optical density measurements. Dormant *B. cereus* spores were sorted with a Beckman Coulter EPICS Elite flow cytometer with 488-nm excitation from a 15-mW argon laser into sterile 96-well microtiter plates (flat-bottom; Greiner Bio-One, the Netherlands). Dormant spores were distinguished from germinated spores by using the fluorescent reporter dye SYTO9 (Invitrogen, the Netherlands) as described previously (18), with the exception that a final concentration of 2 µM dye was used. Plates were closed, subsequently sealed with Parafilm M laboratory film, and stored at -20°C until use (pilot experiments with BHI at pH 7 showed that long-term storage at -20°C did not affect the germination properties of single spores). Before use, each plate was thawed at room temperature for 10 min, and in the experiments with heat treatment, plates were incubated for 10 min at 70°C. After thawing and/or heat treatment, the plates were kept on ice while 200 µl aliquots of bBHI at either pH 7 or 5.5 or at pH 5.5 with supplementation with 0.75 mM HSA were added to each well. The plates were incubated at 30°C in a spectrophotometer (VersaMax; Molecular Devices, United States) for up to 72 h, and during this period, the optical density was measured every 10 min at a wavelength of 600 nm.

A lower pH of 5.5 resulted in more heterogeneity in germination and outgrowth compared to the optimal growth condition, and this heterogeneity was even larger in the presence of 0.75mM HSA (Fig. 2A, B, and C). For the latter stress condition, no growth was detected in approximately 40% of the wells within the time frame of the experiment, underlining that it is conceivable that the observed heterogeneity is underestimated. Heat shock did not decrease the percentage of wells in which no growth was detected and only seemed to have an impact on heterogeneity when spores were exposed to 0.75 mM HSA at pH 5.5 (Fig. 2F). To assess

PhD Thesis Clint van Melis_corrected1.indd 86 4-12-2013 10:55:55

the observed heterogeneity in more detail, the first time point at which each well reached the OD600 value of 0.2 (which equals 2 times the background signal) was calculated and defined as the time to detection. These times to detection were used to visualize the heterogeneity in spore germination and outgrowth (Fig. 3). The distributions became more spread and skewed to the right under more severe stress. Lowering the pH to 5.5 and addition of HSA at pH 5.5 increased both the means and the associated standard deviations of the time-to-detection distributions and also increased the coefficient of variation (Table 1), showing that, relatively, variability had been increased with increasing stress conditions. To test the significance of these observations, the data were normalized with the mean per data set using the formula normalized value = value/mean, and the variances were compared using Levene's test. Levene's test confirmed that germination and outgrowth at pH 5.5 and at pH 5.5 plus 0.75 mM HSA significantly increased the variability compared to optimal conditions ($P<0.05$). In addition, heat treatment of spores

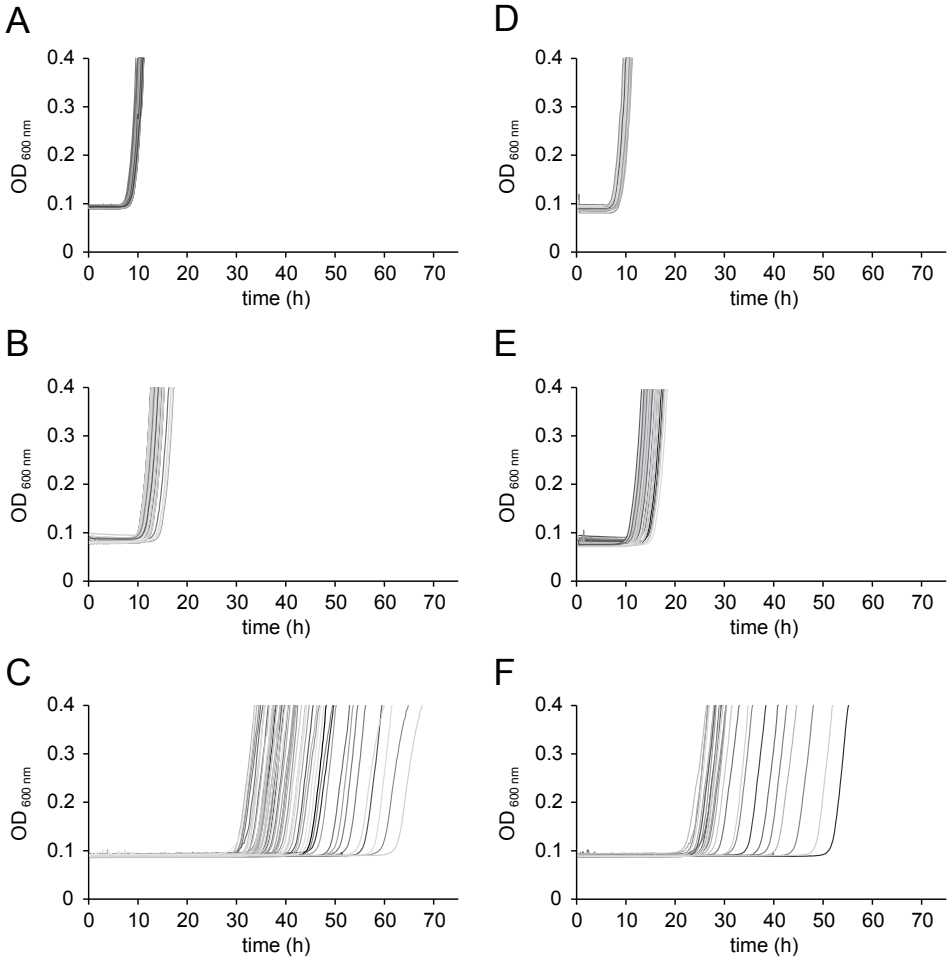

Fig. 2: Heterogeneity in outgrowth initiated from single dormant spores. Before initiation of germination, spores were not heat-shocked (a, b, c) or heat-shocked for 10 min at 70°C (d, e, f), after which germination and outgrowth was monitored at 30°C in buffered BHI at pH 7 (a, d), pH 5.5 (b, e), and pH 5.5 supplemented with 0.75 mM undissociated sorbic acid (c, f). The figures are representative examples of growth curves generated in 96 wells-microtiter plates.

PhD Thesis Clint van Melis_corrected1.indd 87 4-12-2013 10:55:55

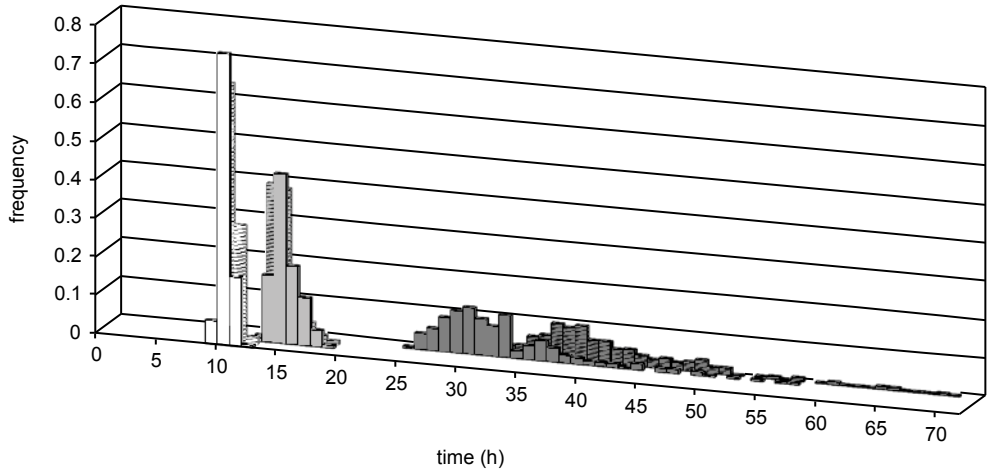

Fig. 3: Frequency distribution of times-to-detection (OD 600 nm = 0.2) initiated from single dormant spores that were not heat-shocked (dashed bars) or heat-shocked for 10 min at 70°C (undashed bars) and incubated at 30°C in buffered BHI at pH 7 (white bars), pH 5.5 (light gray bars), and pH 5.5 supplemented with 0.75 mM undissociated sorbic acid (dark gray bars).

indeed only reduced the variability when spores were subsequently exposed to 0.75 mM HSA at pH 5.5. This reduced heterogeneity in germination and outgrowth kinetics, introduced by a relatively mild heat pretreatment of spores, is in line with previous findings in other spore-formers where mild heat treatment of spores made the distributions smaller in *Bacillus subtilis* (9), whereas more severe lethal heat treatment widened the distribution not only for *B. subtilis* (9), but also for *Clostridium botulinum* (14). We demonstrated now for *B. cereus* that mild heat pretreatment only affected the heterogeneity in outgrowth when spores were exposed to rather stressful conditions, like 0.75 mM HSA at pH 5.5. This suggested that mild heat-induced acceleration of germination at pH 7 and 5.5 (Fig. 1) did not significantly affect heterogeneity in outgrowth performance (Fig. 2 and 3). Additionally, although a rather high germination efficiency was observed in the presence of 0.75 mM HSA—the maximum OD drop corresponded with approximately 95% of germinated spores—the heterogeneity in outgrowth was substantial (Fig. 3) and might even be underestimated as approximately 40% of the wells did not reach the turbidity detection threshold. These observations underlined that germination efficiency did not seem to be a good predictor for heterogeneity in final outgrowth. The observed heterogeneity is the sum of heterogeneity during germination, the first doubling phases, and further outgrowth and is likely to result from multiple sources (6, 15). For C. botulinum, it has been shown that the first spore that germinated was not the first to develop into a vegetative cell, also under stress conditions, highlighting the lack of correlation between germination and outgrowth times (12–14, 19). The most important source of variability in outgrowth might depend on the relative magnitude of the contributions of the different phases in outgrowth. The positively skewed distributions we observed for *B. cereus* spore development under acid stress conditions were similar to the shapes of distribution previously reported for C. botulinum spore germination and outgrowth under stressful conditions (1, 13). This implies that when mean outgrowth times are used in risk assessment, the number of spores that have shorter development times will be underestimated, resulting in a "fail-dangerous" scenario. Heterogeneity in germination

PhD Thesis Clint van Melis_corrected1.indd 88 4-12-2013 10:55:56

Table 1: Effect of heat treatment and subsequent acid stress exposure on heterogeneity in outgrowth. Times-to-detection (OD 600 nm = 0.2) were generated for single dormant spores that were not heat-shocked or heat-shocked for 10 min at 70°C (HS) and incubated in buffered BHI at pH 7, pH 5.5, and pH 5.5 supplemented with 0.75 mM undissociated sorbic acid (HSA)

		Time to detection (h)[b]		
Condition(s)	No. of wells[a]	Mean	SD	Coefficient of variation
pH 7	509	9.9	0.45	0.045
pH 7, HS	331	9.7	0.44	0.045
pH 5.5	563	13.5	1.06	0.079
pH 5.5, HS	305	14.0	1.11	0.079
pH 5.5, 0.75mM HSA	406	39.3	7.85	0.199
pH 5.5, 0.75mM HSA, HS	191	31.5	5.91	0.188

[a]Number of monitored wells that reached time to detection

[b]Times to detection (OD600, 0.2) were generated for single dormant spores that were not heat shocked or were heat shocked for 10 min at 70°C (HS) and incubated in buffered BHI at pH 7 or 5.5 or at pH 5.5 with supplementation with 0.75 mM undissociated sorbic acid (HSA).

and outgrowth complicates their predictability, and recent publications suggest that the levels of germination receptors might vary significantly between spores in a population (20, 21) and might contribute to heterogeneity in performance. We demonstrated that mild acid preservation will increase the heterogeneity in outgrowth kinetics of dormant spores. The next step will be to correlate germination and outgrowth kinetics to spore physiology at the single-cell level, which will provide valuable mechanistic understanding of sources of spore germination and outgrowth heterogeneity. Quantitative information and mechanistic knowledge on heterogeneity in germination and outgrowth will give relevant insights to optimize application of mild preservation factors to control and balance food quality and food safety.

PhD Thesis Clint van Melis_corrected1.indd 89 4-12-2013 10:55:56

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PhD Thesis Clint van Melis_corrected1.indd 91 4-12-2013 10:55:57

PhD Thesis Clint van Melis_corrected1.indd 92 4-12-2013 10:55:57

Chapter 6

Quantification of the impact of single and multiple mild stresses on outgrowth heterogeneity of *Bacillus cereus* spores

C. C. J. van Melis, H. M. W. Den Besten, M. N. Nierop Groot, and T. Abee

Submitted for publication

PhD Thesis Clint van Melis_corrected1.indd 93 4-12-2013 10:55:57

Outgrowth heterogeneity of bacterial spore populations complicates both prediction and efficient control of spore outgrowth. In this study, the impact of mild preservation stresses on outgrowth of *Bacillus cereus* ATCC 14579 spores was quantified during the first stages of outgrowth. Heterogeneity in outgrowth of heat-treated (90°C for 10 min) and non-heat-treated germinated single spores to the maximum micro-colony stage of 256 cells was assessed by direct imaging on Anopore strips, placed on BHI plates at pH 7 and pH 5.5, without and with added NaCl or sorbic acid (HSA). At pH 7 non-heated and heat-treated germinated spores required 6 h to reach the maximum microcolony stage with limited heterogeneity, and these parameters were only slightly affected with both types of spores when incubated at pH 7 with added NaCl. Notably, the most pronounced effects were observed during outgrowth of spores at pH 5.5 without and with added NaCl or HSA. Non-heat-treated germinated spores showed again efficient outgrowth with limited heterogeneity reaching the maximum microcolony stage after 6 h at pH 5.5, which increased to 12 h and 16 h with added NaCl and HSA, respectively. In contrast, heat-treated spores displayed a strong delay between initial germination and swelling and further outgrowth at pH 5.5, resulting in large heterogeneity and low numbers of fastest growers reaching the maximum microcolony stage after 10, 12 and 24 h, without and with added NaCl or HSA, respectively. This work shows that Anopore technology provides quantitative information on the impact of combined preservation stresses on outgrowth of single spores, showing that outgrowth of germinated heat-treated spores is significantly affected at pH 5.5 with a large fraction of spores arrested in the early outgrowth stage, and with outgrowing cells showing large heterogeneity with only a small fraction committed to relatively fast outgrowth.

Introduction

The food industry's current trend of processing under milder conditions and reduced use of preservatives may provide opportunities for spore forming bacteria, such as the food pathogen and spoilage bacterium *Bacillus cereus* (Logan, 2012; Stenfors Arnesen et al., 2008; Ehling-Schulz et al., 2004; Andersson et al., 1995). Dormant spores are resistant to a variety of environmental stress factors, but if appropriate nutrient or non-nutrient germinants are present or applied, they can return to life through germination followed by outgrowth (Setlow, 2003; Abee et al., 2011). Germination of a dormant spore population often occurs as a heterogeneous process (Eijlander et al., 2011; Stringer et al., 2011; Barker et al., 2005), i.e., not all spores will germinate at the same time, nor will transition to vegetative cells and subsequent outgrowth occur at the same rate. Variability in spore behavior (Yi and Setlow, 2010; Stringer et al., 2005) complicates the prediction of outgrowth considerably, since it requires extensive knowledge on the responses of individual spores. Spoilage of products may arise from just a few spores that survived processing and therefore the presence of just a few individual spores in the population with enhanced survival properties can be a challenge to the industry. At the population level, this variability will remain unnoticed and therefore this information cannot be obtained by conventional plate counts or optical density based methods. It is therefore important to study heterogeneity by quantifying outgrowth at the single spore level.

PhD Thesis Clint van Melis_corrected1.indd 94 4-12-2013 10:55:57

Heterogeneity in germination and outgrowth may be further increased by the application of (mild) preservation methods, such as milder heating regimes that do not fully inactivate the spore population, leaving surviving (partially damaged) spores. Thus, less intensive heating should therefore be combined with other preservation 'hurdles', including for example the addition of organic acids, low concentrations of salts, or combinations thereof, in order to control germination and outgrowth of surviving spores in food products. Quantification of the impact of stress exposure on population heterogeneity has been presented for heat treatment of *B. cereus* spores (Cronin and Wilkinson, 2008), *Bacillus subtilis* (Smelt et al., 2008; Pandey et al., 2013) and *Clostridium botulinum* (Stringer et al., 2011) and heat and/or salt-stressed *C. botulinum* spores (Webb et al., 2007). We previously used flow cytometry sorting to monitor germination and outgrowth of single dormant spores and quantified acid-induced population heterogeneity at pH 5.5 without and with added sorbic acid. Evidence was presented that *B. cereus* ATCC14579 spore germination efficiency was not a good predictor for heterogeneity in final outgrowth (den Besten et al., 2012).

This underlines the importance to quantify the first stages of (stressed) outgrowth, directly following after the germination phase. Anopore technology was first described by Ingham et al. (2005), and has been used to quantify *Bacillus cereus* ATCC 14579 population heterogeneity at a low incubation temperature and under salt stress conditions (den Besten et al., 2007, 2010). We now used Anopore to monitor the outgrowth of individual non-stressed and heat-treated spores at neutral and slightly acidic pH (pH 7 and pH 5.5) without and with added NaCl (2.5 % w/v) or HSA (0.25 mM undissociated sorbic acid). Behavior of germinated spores was followed up to the microcolony stage (maximum 256 cells), enabling quantification of heterogeneous outgrowth of *B. cereus* spores in the absence and presence of preservation stresses.

Materials and Methods

Strain and culture conditions

Bacillus cereus strain ATCC 14579 was obtained from the American Type Culture Collection (ATCC) and stored in Brain Heart Infusion (BHI) broth, supplemented with 50% glycerol at -80°C. *B. cereus* cells were cultivated at 30°C with aeration at 200 rpm (Innova 4335 Incubator Shaker, New Brunswick Scientific, United States). Overnight cultures were used to inoculate defined minimal sporulation medium (De Vries et al., 2004) and spores were produced and harvested as described previously (Van Melis et al., 2011) with the following modification: the Tween 80 content was reduced from 0.1% to 0% by daily washing steps over a period of five days. Pure spore crops devoid of vegetative cells and debris were stored in phosphate-buffered saline (MES, pH7) at 4°C for not more than 8 weeks until use.

Outgrowth percentages on agar plates

To select a heating regime leading to sub-lethally damaged spores, a stock of dormant spores ($2x10^8$ spores/ml) was ten times diluted in MES buffer and exposed to various time/temperature combinations in Eppendorf tubes at 85, 90, 95 and 100°C for either 10 or 20 minutes. After treatment, the spore solutions were further diluted and plated on BHI plates that were buffered at pH 7 and contained either 0 or 1.5% (w/v) of added NaCl to determine the fraction of sub-lethally damaged spores, since these will not produce colonies in the latter condition whereas

PhD Thesis Clint van Melis_corrected1.indd 95 4-12-2013 10:55:57

they will in absence of added NaCl. The heat treatment of 90°C for 10 minutes was selected for further experiments since this heat treatment induced the highest fraction of sub-lethally damaged spores (data not shown).

To assess the colony forming capacity of heated and non-heated spores in the presence of mild stresses, dilutions were made in MES buffer (pH 7 or pH 5.5, depending on the test conditions), and plated on buffered BHI plates containing 0 or 2.5% (w/v) NaCl (at both pH 7 and pH 5.5), or 0.25 mM undissociated sorbic acid (HSA, only at pH 5.5). Colonies were counted after an incubation period of 24 hours at 30°C, and again after 48 hours. Quantification of colony forming spores was performed in three individual experiments (biological triplicates), each of which consisted of technical duplicates, resulting in a total number of six determinations per condition. The numbers of colonies formed by non-heated spores at pH 7 were averaged, and this value was set as 100%, and the number of colonies of the other stress-conditions are expressed relatively to those observed for non-heated spores.

Growth on Anopore strips

Anopore strips (Whatman, The Netherlands) were prepared as described previously (den Besten et al., 2007; 2010). Sterile Anopore strips were placed on pre-warmed (30°C) BHI plates (buffered at either pH 7 or pH 5.5), supplemented with 2.5% NaCl (w/v) or, in case of plates with pH 5.5, with 0.25 mM undissociated sorbic acid (HSA, total concentration SA of 1.63mM). Anopore strips were inoculated with non-heated and heat-treated spores derived from a dormant spore stock (2x108 spores/ml) appropriately diluted in MES buffer of either pH 7 or pH 5.5, depending of the test conditions. The plates were closed and then incubated at 30°C in a moist chamber. For each imaging time point, one Anopore strip was transferred right side up to a microscope slide (50x76x1 mm) covered with a 1mm thick film of 1% (w/v) solidified low-melting-point agarose (Invitrogen, The Netherlands). The agarose was dissolved in MES, buffered at the appropriate pH, and 2.5 µM SYTO-9 (Invitrogen, The Netherlands) was added to stain the micro-colonies on the Anopore strip. The micro-colonies were then incubated for 15 minutes at room temperature in the dark before imaging.

Anopore strips were imaged directly (i.e. without the use of a coverslip and immersion oil) using an Axioskop fluorescence microscope equipped with a LD-Plan Neofluar 63x 0.75Ph 2 Corr lens (Zeiss, The Netherlands) and Zeiss type 38 filters. An Olympus XC30 camera, controlled by Olympus Cell B imaging software, was used to capture the images. The images were analyzed as described previously (den Besten et al., 2007; 2010). The fluorescence-based approach of Anopore allowed to monitor fluorescent spores as soon as these germinated and started with the uptake of water resulting in swollen, phase-dark spores. This step was reached after 20 to 30 minutes under all test conditions (data not shown), and 30 minutes was therefore chosen as the starting time point for all subsequent measurements. The intervals of imaging were chosen in such a way that for each stress condition the outgrowth process could be followed, starting from germinated single spores up to microcolonies of 256 cells, after which 2D-based area measurements were no longer possible due to multiple layers of cells being formed in the microcolonies. At each imaging time point, the number of images that were processed was chosen to cover an average of 280 spores/microcolonies per sampling point.

PhD Thesis Clint van Melis_corrected1.indd 96 4-12-2013 10:55:57

Quantification of heterogeneity

Microsoft Excel was used to calculate the distribution of microcolony areas per imaging time point for each experimental condition, and the observed frequency distributions were presented in histograms. To optimally envisage the increase of microcolony area over time, the binning of the histograms was based on the number of cells per microcolony, calculated by dividing the areas of the individual microcolonies by the area of an average single cell, measured under identical stress conditions. This approach proved adequate for microcolonies consisting of vegetative cells, but not for representing the different stages of outgrowing spores. Therefore, all areas that were smaller than that of one cell were grouped together into one bin (represented by the letter S in the figures), representing both germinated and outgrowing spores. The population heterogeneity was expressed by calculating the variance per sampling time-point for each condition.

Results

Impact of mild stresses on colony formation of spores

Comparison of colony formation capacity of non-heated and heat-treated spores (10 min at 90°C) showed similar results at pH 7 (Fig. 1). However, heat-treated spores were significantly impaired in colony formation at pH 7 with 2.5 % (w/v) added NaCl, and at pH 5.5 without and with 2.5 % (w/v) added NaCl or sorbic acid (0.25 mM undissociated sorbic acid; HSA) compared to non-heated spores (Fig. 1). The impact of these stress conditions on population heterogeneity in the early outgrowth phase of non-heated and heat-treated spores was subsequently quantified using Anopore.

Quantification of population heterogeneity by monitoring outgrowth of single spores on Anopore

To study spore outgrowth heterogeneity in more detail, we quantified the behavior of non-heated and heat-treated spores on Anopore strips at pH 7 and pH 5.5 without and with additional mild stresses. This allowed us to quantify population heterogeneity at the early stages of spore

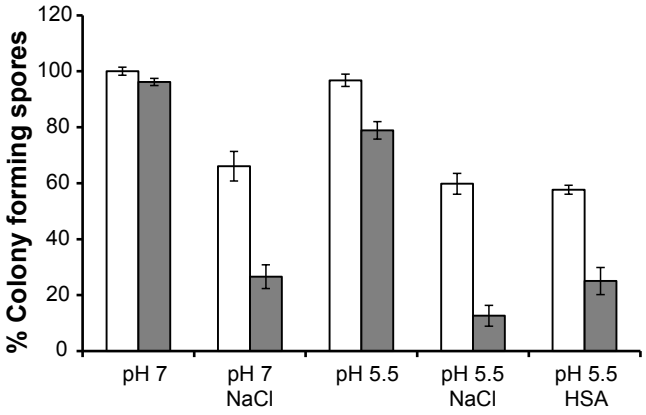

Fig. 1: Impact of mild stresses on colony formation of non-heated and heat-treated *B. cereus* ATCC 14579 spores. The spores were either untreated (white bars) or heat-treated for 10 minutes at 90°C (dark bars). The test-conditions in the BHI plates are indicated under the bars, and include pH 7 without and with 2.5% NaCl (w/v), and pH 5.5 without and with 2.5% NaCl (w/v) or sorbic acid (0.25mM undissociated sorbic acid; HSA). The number of colonies formed by untreated spores at pH7 was set as 100%, and all the other percentages are relative to this percentage. Results represent the averages of biological triplicates.

PhD Thesis Clint van Melis_corrected1.indd 97 4-12-2013 10:55:57

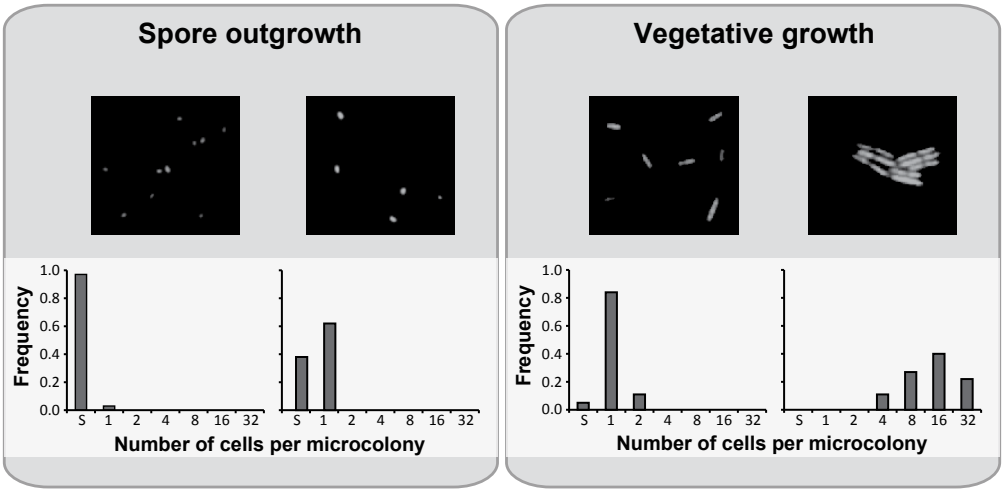

Fig. 2: Schematic overview for quantifying heterogeneity in outgrowth of single spores. The example images show fluorescence microscope captures of SYTO-9 stained, germinated phase dark spores and microcolonies on Anopore strips, after incubation on BHI (pH7) at different time intervals. Bars show examples of the corresponding frequency distributions of the number of cells per microcolony for each time point. The numbers of each bin on the x-axis represent the number of cells per microcolony up to bin 32 is shown (maximum microcolony size is 256 cells; bin256), and bin 's' represents germinated (phase dark) spores.

outgrowth, starting from germinated phase dark spores to microcolonies that consisted of a maximum of 256 cells (exemplified in Figure 2, with microcolonies up to 32 cells). The time to germinate was not considered in this approach, since more than 95% of control and heat-treated spores germinated within 30 min in all tested conditions as determined by phase-contrast microscopy and flow cytometry (data not shown), and previous work by our group and by others (Stringer et al., 2005, 2011; den Besten et al., 2012) suggested that especially the phase dark spore-vegetative cell transition stage and subsequent cell divisions were particularly relevant for the onset of population heterogeneity.

Figure 3 shows the shift in microcolony size in time used to illustrate and quantify heterogeneity in outgrowth kinetics of non-heated and heat-treated spores. Heat treatment only induced a minor effect on outgrowth heterogeneity at neutral pH (Fig 3, dark bars), confirming that under these conditions the spores were able to recover from any damage induced by the heat treatment. In contrast, outgrowth of these heat-treated spores at pH 5.5 (Fig. 3, panel B) showed to be highly heterogeneous. Although more than 95% of the spores germinated rapidly, a significant delay occurred in the swelling stage (represented in bin S), and only a small fraction of the population was able to resume outgrowth rapidly, with a trend towards reduced outgrowth speed when compared to the non-heated spores. The percentage of spores delayed in the stage before swelling gradually decreased in time, illustrating a large heterogeneity in outgrowth, which compromises the reliability of predictions based on the overall behavior of a population.

To illustrate the impact of single and multiple mild stresses on outgrowth heterogeneity of non-treated and heat-treated spores, we selected the time points at which microcolonies of 256 cells (representing the maximum of eight generations that can be visualized by Anopore) were

PhD Thesis Clint van Melis_corrected1.indd 98 4-12-2013 10:55:57

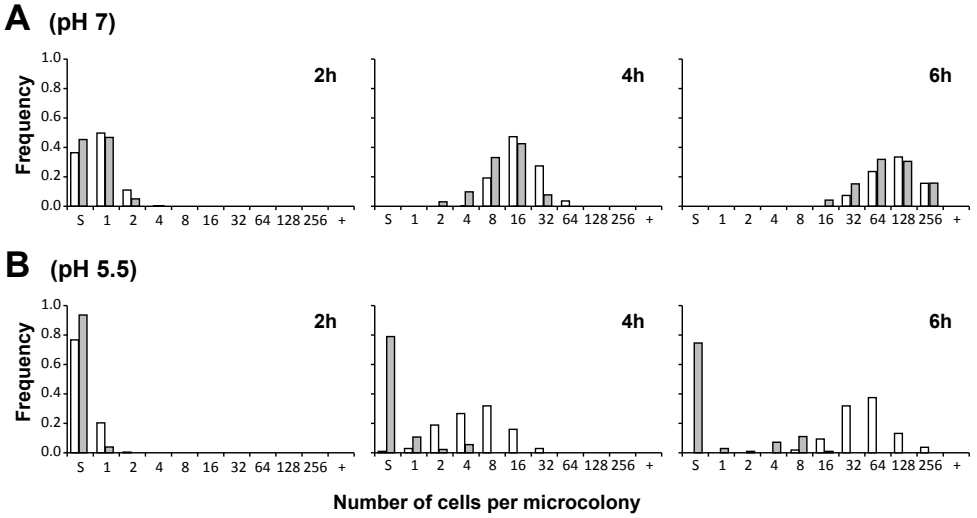

Fig. 3: Frequency distributions of microcolony size of non-heated and heat-treated *B. cereus* ATCC 14579 spores growing out at neutral and mildly acidic pH. Sampling time points are indicated in the right top corner of each distribution histogram. Dormant spores were either directly placed on Anopore strips at the start of experiments (white bars), or heat-treated for 10 minutes at 90°C before being placed on Anopore strips (grey bars) on BHI plates at pH7 (panel A) or pH5.5 (panel B). The numbers for each bin on the x-axis represent the number of cells per microcolony and 's' indicates germinated (phase dark) spores. Distributions are based on 200-300 individual spores/microcolonies per condition.

reached (Fig. 4). A large variation was observed in the incubation times required to reach the latest measureable time point, with a trend towards longer incubation times when spores were exposed to higher levels of stress, i.e., especially mildly acidic pH 5.5 without and with added NaCl or HSA. In addition to a delay in outgrowth, imposed stress(es) during outgrowth also resulted in wider distributions of microcolony sizes, representing a stress-induced increase in outgrowth heterogeneity. The impact on heterogeneity was quantified by calculating the variances for each distribution (Fig. 5). Heat treatment of spores in combination with stress(es) during outgrowth, i.e., either pH 5.5 without and with NaCl or HSA, had a strong effect on heterogeneity, while these stresses individually only displayed a significant effect on heterogeneity in outgrowth of the non-heated spore population. Germinated heat-treated spores displayed an extended lag phase at pH 5.5, i.e., a variable length of time wherein the spores reached the vegetative cell stage (bin 1), before continuing outgrowth (bins 2-256). With increasing incubation times the number of spores that remained in this bin 1 gradually decreased, resulting in wide distributions with low numbers of representatives in each of the different size bins. Notably, with heat-treated spores at pH 5.5 a significant fraction of the spores is maintained in the germinated state (bin S) within the time frame of the experiment. This increased heterogeneity becomes even more noticeable when the variances for these stress conditions are considered (Fig. 5, dark bars). Obviously, outgrowth efficiency and heterogeneity of heat-treated spores is most severely affected upon exposure to pH 5.5, with only minor additional effects of added NaCl or HSA.

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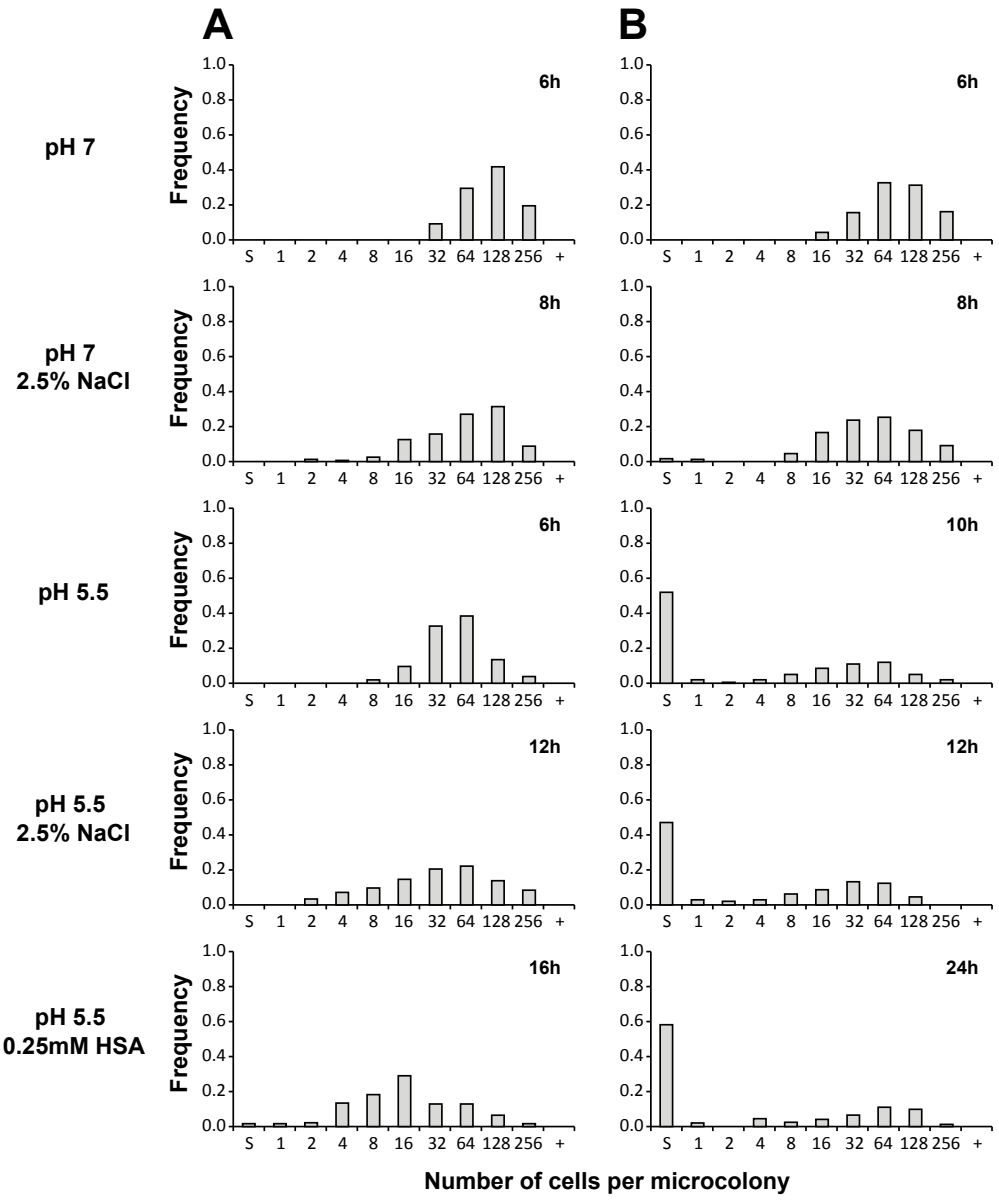

Fig. 4: Frequency distributions of the number of cells per microcolony of non-heated (A) and heat-treated (B) *B. cereus* ATCC 14579 spores incubated under selected (stress-) conditions. Spores were incubated on Anopore strips placed on BHI plates at pH 7 or pH 5.5, without or with added 2.5 % NaCl (w/v) or 0.25mM HSA. The samples represent latest measureable time-points where the fastest growing micro-colonies consisted of 256 cells. The sampling time points are indicated in the right top corner of each distribution histogram. The numbers for each bin on the x-axis represent the number of cells per microcolony and 's' indicates germinated (phase dark) spores. Distributions are based on 200-300 individual spores/microcolonies per condition.

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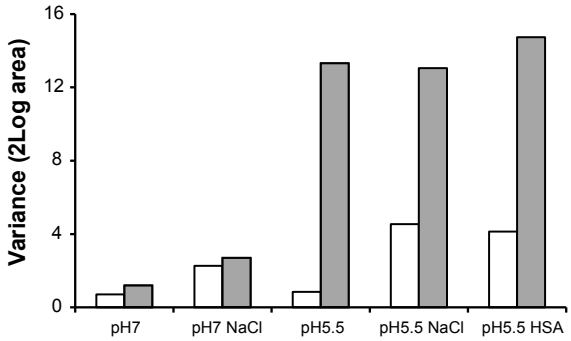

Fig. 5: Variances of frequency distributions at maximum microcolony size of 256 cells for non-heated (white bars) and heat-treated spores (dark bars) at pH 7 and pH 5.5 without and with additional mild stresses. Spores were incubated on Anopore strips placed on BHI plates at pH 7 or pH 5.5, without or with added 2.5 % NaCl (w/v) or 0.25mM HSA. Variances are given for the different conditions at their end point measurements, i.e., the time points where the fastest growing microcolonies consisted of a maximum of 256 cells.

Discussion

Spores of some *Bacillus* species are major agents of food spoilage and food-borne disease and are extremely resistant to a range of stresses including wet heat treatments (Setlow, 2006). Mild heat treatments and/or exposure to single or multiple mild stresses could affect the outgrowth heterogeneity of microbial spores, which in turn complicates the prediction of outgrowth behavior. More accurate prediction of spore behavior requires enhanced understanding of the impact of (combinations of) stresses on germination and outgrowth heterogeneity within spore populations. This also includes the fraction of spores that perform the fastest outgrowth and that could reach spoilage levels affecting the targeted shelf life resulting in loss of product quality and compromising product safety in case of food-borne pathogens such as *Bacillus cereus*. Previous studies on heterogeneity in germination and growth of *Bacillus* spores, using techniques such as flow cytometry (Cronin and Wilkinson, 2008), Raman spectrometry (Zhang et al., 2010; Wang et al., 2011, 2012; Yi & Setlow, 2010) and/or optical density-based monitoring of single spores (Broussolle et al., 2008; Smelt et al., 2008; den Besten et al., 2012) focused in particular on either the germination phase or long term outgrowth. In order to quantify spore population heterogeneity including the behavior of the fastest outgrowing spores, it is also important to quantify outgrowth kinetics of spores during the early stages of (out)growth. In this study, Anopore technology enabled us to quantify heterogeneity during the early stages of outgrowth of non-heated and heat-treated *B. cereus* spores at pH 5.5 and pH 7 without and with additional stresses during outgrowth.

Both non-heated and heat-treated (90°C for 10 min) *B. cereus* ATCC14579 spores showed efficient germination and outgrowth with limited outgrowth heterogeneity at pH 7, but in the presence of additional stresses outgrowth heterogeneity was increased. The most pronounced effects were observed with heat-treated spores incubated at pH 5.5 without and with added NaCl or sorbic acid. The most remarkable effect was the strong delay in outgrowth of a large fraction of the germinated spore population at pH 5.5 without and with additional NaCl or HSA stress. Recently, Pandey et al. (2013) used time-lapse microscopy to assess the influence of a heat stress

PhD Thesis Clint van Melis_corrected1.indd 101 4-12-2013 10:55:58

of 85°C for 10 min on germination, outgrowth, and subsequent vegetative growth of heat-activated (70°C for 30 min) *B. subtilis* 168 spores in MOPS medium supplemented with 10 mM L-asparagine, 10 mM glucose, 1 mM fructose, and 1 mM potassium chloride (AGFK, pH 7). These authors showed that compared to control samples fewer heat-stressed spores germinated (41.1% less) and fewer grew out (48.4% less). The heat treatment significantly increased the average time to the start of germination and also the distribution and average of the duration of germination itself had increased. However, the distribution and the mean outgrowth time and the generation time of vegetative cells, emerging from untreated and thermally injured spores, were similar. Obviously, the use of *B. cereus* versus *B. subtilis* spores, and differences in heat (in) activation treatments and media used, may have contributed to differences observed in inactivation and germination efficiency, and outgrowth heterogeneity in the current study and that of Pandey et al. (2013). The impact of mild acidic pH without and with additional stresses on germination and outgrowth of *B. subtilis* spores remains to be investigated.

Notably, in our study, heat-induced damage to *B. cereus* spores is rapidly repaired during the initial outgrowth phase at pH 7, whereas this process is apparently severely hampered during outgrowth at pH 5.5. Research by Coleman et al. (2010) on wet heat inactivation of *B. cereus* T and B. megaterium QMB1551 spores at 88°C for 30, 60 and 90 min, showed their data to be consistent with the previously presented data for wet heat killing of *B. subtilis* spores (Coleman et al. 2007). Based on the absence of significant ATP production by DPA-replete but wet heat-killed spores of multiple *Bacillus* species these authors suggested that it is through damage to some key enzyme of intermediary metabolism that wet heat kills spores (Coleman et al., 2007, 2010). Our observations on outgrowth of heat-treated *B. cereus* spores at pH 7 compared to that at pH 5.5, suggest that one or more key enzymes and/or processes including damage repair, are specifically affected and/or cannot be repaired in the initial phase after germination at mildly acidic pH. It should be noted that we used a heat treatment (90°C for 10 min) different to that in the studies of Coleman et al. (2007, 2010), where a slightly lower temperature but significantly longer exposure times were used, and this resulted in a high percentage of heat-killed spores and a very low fraction of spores that ultimately showed outgrowth. Whether similar mechanisms are affected in mild heat-damaged *B. cereus* spores as in more severe heat-damaged *Bacillus* spores as proposed by Coleman et al. (2007, 2010), remains to be elucidated.

Obviously, outgrowth of *B. cereus* spores under multiple stress conditions amplified the heterogeneity inherent in both the non-heated and heat-treated spore population, as well as reducing the outgrowth speed of (part of) the spore population. This on one hand supports the hurdle technology concept (Leistner, 2000) where multiple, simultaneously applied mild stresses can be used to inhibit (out)growth, but our data also highlight that enhanced heterogeneity can complicate the prediction of initial outgrowth behavior of *B. cereus* spores. While the importance of the germination phase in population heterogeneity has been referred to previously (among others: Brousolle et al., 2008; Cronin & Wilkinson, 2008; Webb et al., 2007), the present study points to an important role of the initial outgrowth phase in enhancing population heterogeneity since both in the current study (data not shown) and in previous work (van Melis et al., 2011) we observed germination percentages of ≥95% under the selected stress conditions. Thus, the current study confirms our previous observation that germination efficiency may not

PhD Thesis Clint van Melis_corrected1.indd 102 4-12-2013 10:55:58

be a good predictor for outgrowth heterogeneity (den Besten et al., 2012). Studies on *C. botulinum* spores have hinted at similar implications, showing that the first spore to germinate is not necessarily the first spore to initiate outgrowth, emphasizing the lack of correlation between germination and outgrowth (Webb et al., 2007; Stringer et al., 2005, 2011). Obviously, a good understanding of damage and repair mechanisms in heat-treated spores is required to understand and predict outgrowth behaviour under mild stress conditions and combinations thereof. Such insights may further add to rational designs in hurdle technology. The next step would be to expand our observations on *B. cereus* ATCC14579 to the quantification of outgrowth heterogeneity of other spore formers, including industrial isolates, and using food-based media.

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Chapter 7

Discussion and future perspectives

PhD Thesis Clint van Melis_corrected1.indd 107 4-12-2013 10:55:58

1. Discussion

With the current demand for less intensive processing of food products, opportunities arise for spoilage and pathogenic microorganisms in food. Approaches include the use of milder heating regimes, and it is especially the spore-forming bacteria that may survive under these conditions. In order to guarantee the quality and safety of products, additional (mild) 'hurdles' (Leistner, 2000) are required to control (out-)growth of these organisms. Weak organic acids are commonly used components that are used as hurdles in preservation of food products. Of particular interest is sorbic acid, which is frequently used because of its effective inhibition of a diverse set of microorganism including bacteria, yeast and fungi. Its mode of action appears to be different from that of other commonly used organic acids such as acetic acid. These differences in mode of action are related to its presumed accumulation in membranes facilitated by the lipophilic character of the undissociated acid at (mildly) acidic pH (Chu et al., 2009; Stratford and Eklund, 2003; Brul et al., 2002; Stratford and Anslow, 1998; Eklund, 1983; Yasudo-Yasaki et al., 1978). The project described in this thesis was initiated to assess the impact of sorbic acid and other mild preservation stresses on the germination and outgrowth of *Bacillus cereus* spores. The project focused on two main aspects of spore outgrowth: 1) the inhibitory effect of sorbic acid on spore germination and outgrowth and the role of its lipophilic character, and 2) the impact of sorbic acid and other (mild) stresses on outgrowth heterogeneity within a *B. cereus* spore population.

The majority of studies on sorbic acid and its mode of action has focused on spoilage organisms that are typically encountered in food products, such as yeasts and moulds (amongst others: Stratford et al., 2013a; 2013b; 2012; Ullah et al., 2013), but more recent studies focus on spore forming bacteria such as *Bacilli* (van Beilen and Brul, 2013; Ter Beek et al., 2008; Chapters 2 – 6 in this thesis). This thesis describes three different approaches used to study the impact of sorbic acid and other mild preservation stresses on spore germination and outgrowth. A transcriptome analysis was performed to assess the response of *B. cereus* spores to low, medium and high concentrations of sorbic acid and gene expression profiles were linked to characterized phenotypes of sorbic acid-exposed spores (Chapter 2). Spore germination was affected by sorbic acid at different stages depending on the concentration applied. At low concentrations, spore germination and outgrowth was only delayed, whereas at medium levels germination was triggered but outgrowth was prevented and germination was completely blocked at high levels of sorbic acid. The work described in Chapter 3 shows that inhibition of germination occurs in a germination receptor-dependent way. The role of the hydrophobic character of sorbic acid in its effectivity was studied in more detail in a comparative analysis of germination inhibition using selected undissociated organic acids of increasing chain length and structurally similar alcohols (Chapter 4). The study described in Chapter 2 pointed to a stress-induced increase in germination heterogeneity. To further analyze the impact of the use of weak organic acids on heterogeneity in spore outgrowth two different approached were used: FACS-assisted sorting of single dormant spores, followed by monitoring of (un)stressed outgrowth (Chapter 5) and Anopore-based quantification of outgrowth heterogeneity on the population level (Chapter 6). An overview of the topics described in this thesis is shown in Figure 1.

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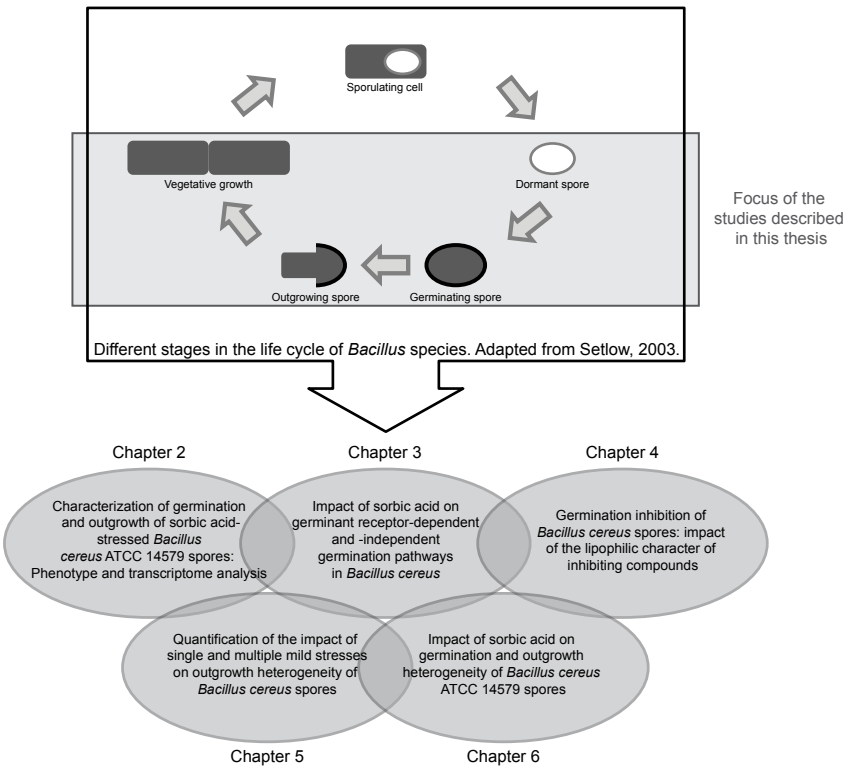

Fig. 1: Overview of the research themes addressed in this study; the impact of organic acids on germination and outgrowth, and outgrowth heterogeneity under preservation stresses. The balloons represent the different research topics that are described in Chapters 2 to 6.

2. Spore-formers: *B. cereus* versus *B. subtilis*

Research on sporulation-, germination- and resistance-mechanisms of spore-forming bacteria, has for many years focused on *B. subtilis* (among others: Higgins & Dworkin, 2012; Setlow, 2006; 2003; Moir, 2006). *B. subtilis* has been widely used as a model organism and a wide array of genetic tools is available, and this species is frequently presented as a representative for the *Bacillus* genera. From more recent work we learned that not all physiological responses and molecular mechanisms in place for *B. subtilis* can be directly translated to other species from the same genus. In fact, large variations can be found between different strains of the same species, both at the genotypic and phenotypic level, affecting characteristics such a metabolic capacity, growth performance, sporulation and germination characteristics (Abee et al., 2011; Earl et al., 2008; Vilas-Bôas et al., 2007; Anderson et al., 2005; Rasko et al., 2005). High diversity in the type of environmental niches that can be occupied has been shown for *B. cereus*, *Bacillus thuringiensis* and *Bacillus anthracis*, even though these organisms are all members of the *B. cereus* group (Ceuppens et al., 2013; Jensen et al., 2003). With the discovery of psychrotolerant *B. cereus* group members (Lechner et al., 1998) this group further diversified. On-going research leads to a growing number of ecological niches that can be occupied by strains of the same spore-forming species and points to the existence of specific mechanisms underlying cellular differentiation including sporulation, and subsequent spore germination and outgrowth.

PhD Thesis Clint van Melis_corrected1.indd 109 4-12-2013 10:55:59

The members of the *B. cereus* group are not very closely related to *B. subtilis* (Bhandari et al., 2013) which is reflected in the genome sizes of both species, with the *B. cereus* ATCC 14579 genome encoding 5502 putative genes (Ivanova et al., 2003) versus the 4422 that have been described for *B. subtilis* 168 (Kunst et al., 1997). Besides the larger repertoire of genes, *B. cereus* has been shown to respond differently to environmental stress. For example, transcriptional regulation of the general stress regulator sigmaB in *B. cereus* differs from that in *B. subtilis* (Scott and Dyer, 2012; Schmidt et al., 2011; Hecker et al., 2007; de Been et al., 2010; 2011). In *B. cereus*, this regulon encompasses a relatively small number of genes as compared to *B. subtilis*, and only a small number of these are shared with *B. subtilis* (van Schaik et al., 2007). The differences in regulatory mechanisms is further illustrated by the high number of transcriptional regulators that belong to the sigma factor-family in *B. cereus*, suggesting a role in fine tuning of the stress response (van der Voort, 2008). Germination of *B. subtilis* PS832 (a derivative of the commonly exploited laboratory strain type 168) spores was inhibited at concentrations of undissociated sorbic acid (HSA) that are a factor 1000 lower than those required to inhibit *B. cereus* ATCC 14579 spore germination (Cortezzo et al., 2004; Chapter 2). However, the inhibition *B. subtilis* by this low amount of sorbic acid appears to block nutrient-induced germination specifically via the GerA germinant receptor and not via AFGK-induced germination. More recent work by Van Beilen et al (2013) using AFGK to trigger *B. subtilis* spore germination shows that substantially higher concentrations of sorbic acid (>0.5 mM HSA) are required to inhibit spore germination. In *B. cereus* HSA concentrations were used that fully block germination (3 mM HSA) but was non-specific for the germinant receptor type (Chapter 3). It remains to be addressed whether the mode of action and involvement of specific receptors is concentration dependent.

Besides the above mentioned examples of interspecies differences, there can also be substantial differences between strains of the same species. As an example, Broussolle et al. (2008) have observed large differences in stress response and outgrowth behaviour between selected *B. cereus* strains, isolated from a range of food-sources and/or food-poisoning outbreaks (Broussolle et al., 2008). These strains displayed high levels of variability in their L-alanine and inosine-mediated germination behaviour, and this variability increased when spores germinated under stress conditions induced by low pH and high NaCl concentrations. Five germinant receptors were shown to be conserved within the majority of the members of the *B. cereus* group (Abee et al., 2011). Four strains from this group, sharing the same core set of these five germinant receptors, had distinct individual requirements for efficient germination, including heat activation, the presence of a co-germinant and/or a high level of specificity for amino-acid germinants (van der Voort et al., 2010), showing that even within a group of closely related strains there can be large differences within germination activation parameters and mechanisms. Obviously, such considerations have to be taken into account and based on possible differences between *B. subtilis* and *B. cereus*, the use of model strains representing the *B. cereus* group is also relevant.

3. The mode of action of lipophilic organic acids on spore germination

Most studies on the mode of action of weak organic acids have focused on vegetative cells, but the impact on spore germination has only received limited attention (Blocher and Busta, 1985; Booth and Stratford, 2003; Cortezzo et al., 2004; Seward et al., 1982; Smigic et al., 2010; Smoot

PhD Thesis Clint van Melis_corrected1.indd 110 4-12-2013 10:55:59

and Pierson, 1981; Wong and Chen, 1988). In this project, the mode of action of sorbic acid and other lipophilic organic acids on spore germination and outgrowth was studied in more detail by using different approaches, described in Chapters 2, 3 and 4. Transcriptome analysis of *B. cereus* ATCC 14579 spores germinating at pH 7 and at pH 5.5, without and with added sorbic acid revealed a limited number of genes to be specifically expressed in response to sorbic acid, including genes involved in modifications of the cell envelope and (multidrug) transporter systems. Sorbic acid-induced modifications of the cell envelope and activation of (specific) transporter systems have previously been observed in *B. subtilis* cells (Ter Beek et al., 2008) and yeasts (Piper, 2011; 2001; Papadimitriou et al., 2007), and this prompted us to investigate if the activation of such systems could also provide protection against sorbic acid in *B. cereus* during (early stages of) spore outgrowth.

A selection was made of the genes that were strongly up-regulated specifically under sorbic acid stress, resulting in four candidate target-genes for construction of deletion mutants: a two-component system (BC3200/3201), drug/metabolite exporter (BC3038), potassium uptake protein (BC3110) and an aminoethyl phosphanate transporter (BC1324/1325). For mutant construction the pAul-A suicide vector harbouring a thermosensitive replicon (Chakraborty et al., 1992) was exploited, resulting in marker-free deletion-mutants of all four selected candidate-genes (data not shown). Spore batches were produced for the four mutants, and these spores were subjected to a series of germination and growth studies at neutral and (mildly) acidic pH in absence and presence of a range of stresses including sorbic acid. Optical density and plate count-based methods were used to monitor germination and outgrowth efficiency and growth rate of the mutant spores, and these were compared with behaviour of the wild type. Unexpectedly, none of the selected mutants displayed an altered sensitivity to sorbic acid. The ΔBC3038 mutant with the deleted drug/metabolite transporter germinated less efficiently at pH 5.5, both in the presence and absence of sorbic acid. Both spore outgrowth and vegetative growth were highly similar between the mutants and the wild type. This illustrates that targeting single sorbic-acid responsive genes does not directly result in a more sensitive phenotype and suggests that expression levels of these genes have limited predictive value for phenotypes of mutants. A low correlation between regulation of genes and phenotype of mutants was also observed by Ter Beek et al. (2009) in *B. subtilis*, where deletion of sorbic acid-responsive genes did not result in more sensitive phenotypes.

Chapter 2 describes the observation that different germination- and outgrowth-phenotypes of dormant *B. cereus* spores at pH 5.5 are in place, dependent on the concentration of HSA. At low concentrations (0.25 – 1.5mM), spore germination and outgrowth was only delayed, whereas at high concentrations, i.e., above 2 mM HSA complete blockage of germination was observed. The transition of phase bright to phase dark spores (corresponding with Ca-DPA release and the first phase of water uptake), did not take place in the presence of high concentrations of HSA, showing that even the initial phase of germination was blocked. In chapter 3, known non-nutrient factors that trigger later germination events in the pathway (Setlow, 2003) were tested on sorbic acid-arrested spores. The experiments performed in this study (described in Chapters 3 and 4) enabled us to make two important observations: i) blockage of germination by sorbic acid and other short chain lipophilic compounds can be relieved by removal or re-

PhD Thesis Clint van Melis_corrected1.indd 111 4-12-2013 10:55:59

duction of the concentration of HSA or alcohol, and spores membranes were not irreversibly damaged or disrupted, and ii) germination inhibition by sorbic acid only affects Germinant Receptor (GR)-mediated germination, directly after the 'germinant binds to the germinant receptor'-step in the germination process. These observations fit into a hypothetical model where HSA or corresponding low molecular weight lipophilic alcohols (chapters 3 and 4) accumulate into the outer layer of the spore's inner membrane and in this way interfere with germination signalling. In the next section the obtained insights will be coupled to current insights in *Bacillus* spore germination (Atluri et al., 2006; Griffits et al., 2011; Li et al., 2012; Setlow, 2003; Vepachedu and Setlow, 2005; 2007; Wang et al., 2011b; Yi et al., 2011).

Although the molecular details of the initiation of spore germination are not fully understood, recent studies involving *B. subtilis* spores have provided more insight in specific roles of several components. Earlier studies have suggested that GerD is located in the spore's inner membrane and, while not essential for germination, plays an important role in rapid germination, either via direct interaction with nutrient receptors and/or as a signal transducer (Pelczar et al., 2007; 2008; Mongkolthanaruk et al., 2009). More recent studies suggest that nutrient germinant receptors are co-localized with GerD proteins in the dormant spore's inner membrane, the so-called germinosome, and that all three subunits of the germinant receptor and GerD are required for this clustering (Griffiths et al., 2011). In their study, Griffiths et al. showed that the absence of clustering resulted in significant reduction or even complete loss of germination potential, suggesting that these protein clusters are functional aggregates that are required for optimal germination. In addition to being located in clusters, a certain level of cooperativity, or synergy, between different nutrient receptors has also been hypothesized (Yi et al., 2011; Atluri et al., 2006). In *B. subtilis*, for instance, Atluri et al. (2006) showed that activation of the GerK GR stimulates L-alanine induced germination via GerA, and GerK is even required for germination via the GerB receptor. The mechanism whereby these germinant receptors cooperate is not known, but given their largely immobile state (Cowan et al., 2004), one can expect that the germinant receptors have to be located in close proximity to each other in order to cooperate, conceivably within the same cluster. This close proximity would be a requirement for signalling (Vepachedu and Setlow, 2007) to the SpoVA proteins that are also located in the spore's inner membrane, and are presumed to form putative SpoVA-channels (Vepachedu and Setlow, 2005; 2007). The spoVA operon encodes six different SpoVA proteins that are involved in transport of Ca-dipicolinic acid (Ca-DPA) chelates into the spore core during sporulation (Li et al., 2012; Tovar-Rojo et al., 2002) and conceivably also in the release of low molecular weight compounds, including Ca-DPA, upon germination of the spore (Wang et al., 2011b). The exact mechanisms of Ca-DPA transport still remains to be elucidated, although recent evidence was presented that SpoVAD is involved in the uptake of Ca-DPA (Li et al., 2012), and SpoVAC appears to be involved in the release of Ca-DPA (Wang et al., 2011b). More recently, spoVAC was suggested to act as a mechanosensitive channel (Velasquez et al, submitted for publication). Further studies are required to specify the exact function of SpoVA proteins including their location in germinosomes, as well as the composition of such germination protein clusters.

With the current knowledge on germination-related protein clusters published by Setlow and co-workers (Griffiths et al. 2011) and the lack of a well-defined model of germination protein

PhD Thesis Clint van Melis_corrected1.indd 112 4-12-2013 10:55:59

arrangement, we can only hypothesize how accumulation of small lipophilic compounds into the spore's inner membrane can interfere with germination and such a hypothetical model is presented in Fig 2. Protein clusters are formed that include GerD and single or multiple germinant receptors, either consisting of one specific receptor or (randomly) mixed groups of different receptors. These clusters are in direct contact, or otherwise in close proximity to, one or more SpoVA channels (Fig. 2). In such a model, accumulation of high amounts of lipophilic compounds, such as HSA concentrations that completely block germination could affect organisation and presumed protein-protein interactions in the germinosome (Fig. 2). It is known that HSA molecules are embedded near the surface of the outer phospholipid layer of the cell membrane, suggesting that interactions between head groups of phospholipids and/or membrane proteins can be affected (Chu et al., 2009). Concentrations of HSA such as were used in the experiments described in Chapter 2 and 3 will occupy a large percentage of the membrane-surface, which may affect functionality of the germinosome including interactions between GR/GerD clusters and SpoVA channels leading to inhibition of germination. In this

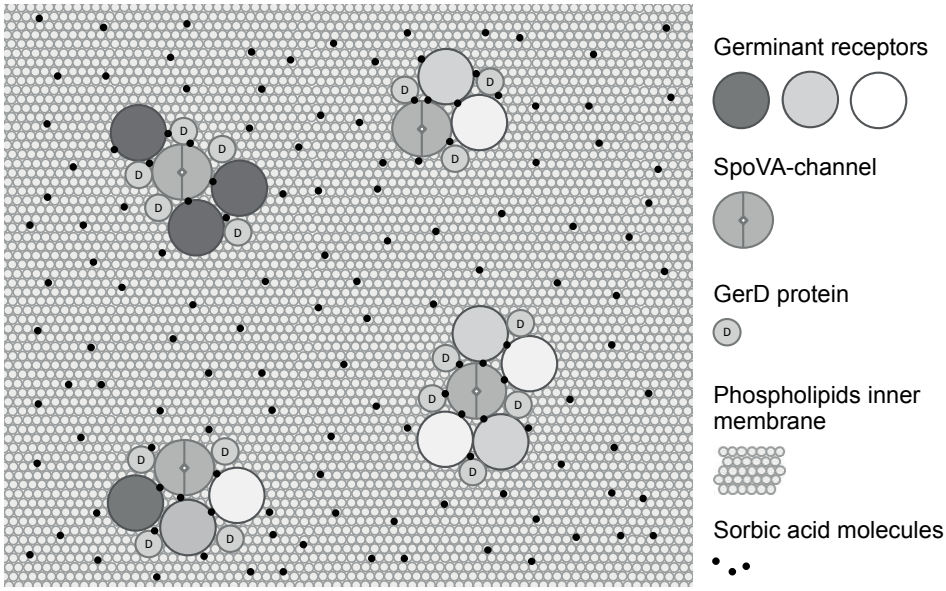

Fig. 2: Hypothetical model of the components making up the germinosome, located in the spore's inner membrane. Germinant receptors (GR) and GerD proteins primarily co-localize to a single cluster in dormant spores, with all three subunits of GRs and GerD being essential for the formation of these clusters (Griffiths et al., 2011). Synergism was observed between the different GRs, where activation of multiple GRs triggered a germination response stronger than the sum of the commitment and germination rates observed with individual germinants (Yi et al., 2011; Atluri et al., 2006). Due to their largely immobile state (Cowan et al., 2004) it can be expected that, in order to cooperate, the GRs are located in close proximity to each other, possibly in mixed clusters of multiple GRs. Also located in the spore's inner membrane are the SpoVA proteins (Vepachedu and Setlow, 2005) that are involved in transport of Ca-dipicolinic acid (Ca-DPA; Li et al., 2012; Tovar-Rojo et al., 2002). Of the six SpoVA proteins, SpoVAC is hypothesized to be involved in the release of Ca-DPA (Wang et al., 2011). Interaction was shown between GRs and SpoVA proteins, presumably for signal transduction through physical contact (Vepachedu and Setlow, 2007). This means that GR/GerD clusters should be localized adjacent to SpoVA-channels. The three components that comprise the GRs are presented as one unit (see text for details). The different components are an artistic top-view representation and are not up to actual shape, scale and/or proportions. The accumulation of molecules of undissociated sorbic acid into the inner membrane is represented by black circles).

PhD Thesis Clint van Melis_corrected1.indd 113 4-12-2013 10:56:02

scenario, germination signalling could be blocked even when the GRs are activated by nutrients. A reduction in the number of accumulated small molecules, such as resulting from changes in the equilibrium between dissociated and undissociated particles, would then allow for recovery of germinosome functionality enabling the initiation of germination. Our observations on reversibility of sorbic acid-arrested germination (Chapter 3), as well as the similarity between sorbic acid-induced inhibition and inhibition by low concentrations (2.5 - 23 mM) of corresponding alcohols such as hexanol and pentanol (Chapter 4), are in agreement with the proposed model. The observation that sorbic acid-arrested germination can be by-passed by extraneous Ca-DPA or high hydrostatic pressure (500 – 600 MPa) as described in Chapter 3 is in line with the model, since these processes (in)directly activate the SpoVA channel without the involvement of GRs (Paidhungat et al., 2002). The fact that germination is still triggered via GR-independent, bryostatin-induced, PrkC pathway and bypasses inhibition by HSA further supports such a model.

The germination pathway of dormant *Bacillus* spores can be initiated by both nutrient and non-nutrient triggers and the stages where HSA can interfere are indicated in Figure 3. In food preservation different approaches are used to control spore-forming bacteria. A recent review by Reineke et al. (2013) provides an overview of current insight in the inactivation of *B. subtilis* spores by wet heat and high hydrostatic pressure, and inactivation by wet heat treatment is also discussed in several papers by Coleman et al. (2010; 2009; 2007). Germination inactivation by wet heat appears to mainly occur through denaturation of key proteins that are relevant for germination. Heat treatment of spores resulted in two subpopulations of spores consisting of spores that lost most of their DPA and those that retained DPA (Coleman et al., 2010; 2007). The spores that lost their DPA had undergone significant protein denaturation and were apparently dead. The second subpopulation retained their DPA, and germinated normally when triggered, but ≥98% of the spores were not able to grow out, possibly due to denaturation of proteins or enzymes that are essential for metabolism including generation of ATP. Inactivation by high hydrostatic pressure is assumed to be a two-step process (Reineke et al., 2013; 2012), involving pressure-induced release of Ca-DPA, accompanied by a loss of heat resistance, followed by inactivation by the pressure and associated temperature conditions. Depending on the pressure intensity and temperature applied, germinant receptors, SpoVA proteins and/or cortex lytic enzymes may be targeted. Combining these observations, several inactivation sites can be visualised in the germination pathway (Fig. 3). The studies described in this thesis (Chapters 2, 3 and 4) add other control/interference-sites to this germination model, including the action of small lipophilic compounds such as HSA and related acids and their corresponding alcohols.

4. The impact of outgrowth heterogeneity on food preservation

Heterogeneity in spore behaviour complicates the prediction of outgrowth, since it requires quantitative information on the responses of individual spores. Spoilage of products may arise from a small number of spores that survived processing and therefore the presence of just a few individual spores in the population with enhanced survival properties can be a cause of concern for the food industry. The use of (mild) preservation methods may even further increase heterogeneity in germination and outgrowth, for example when milder heating regimes are used that do not fully inactivate the spore population, leaving surviving and/or (par

PhD Thesis Clint van Melis_corrected1.indd 114 4-12-2013 10:56:02

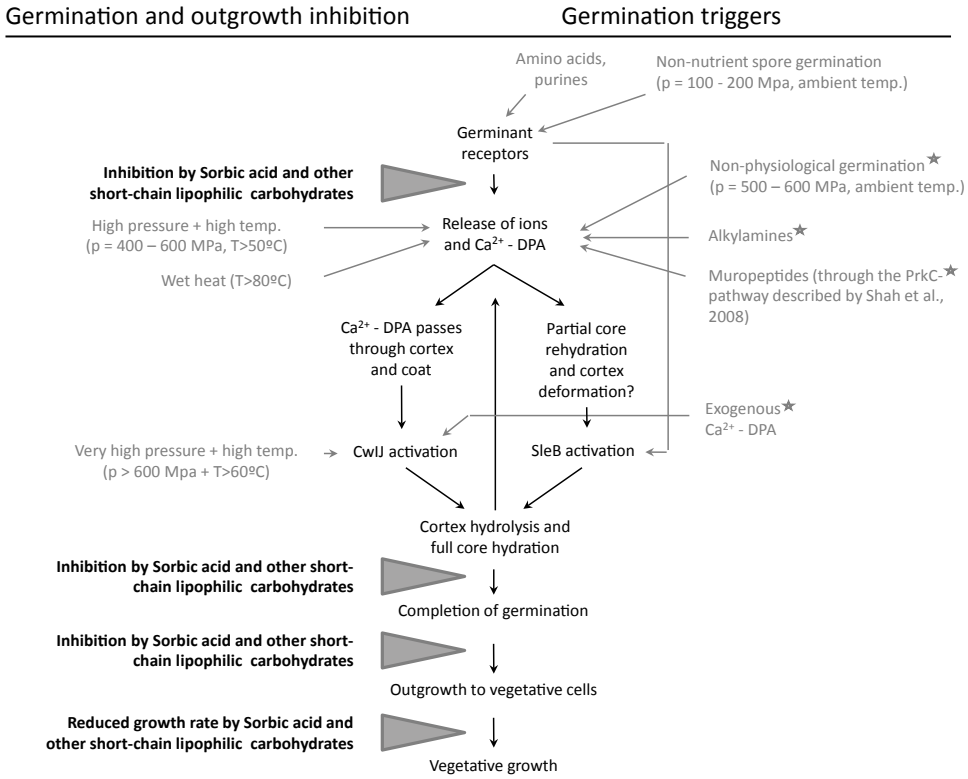

Fig. 3: Impact of sorbic acid in germination and outgrowth of *Bacillus* spp, including germination triggers (shown to the right side of the germination pathway). Grey triangles show where sorbic acid interferes in this pathway. The location of inhibition depends on the concentration of undissociated sorbic acid, with high concentrations (≥ 3mM) completely blocking germination, while lower concentrations allow (partial) germination and/or affect outgrowth and vegetative growth. Complete germination inhibition by high concentrations of undissociated sorbic acid can be bypassed with the germination triggers that are indicated with a star. (Adapted from Setlow, 2003).

tially) damaged spores. To enable more accurate prediction of spore behaviour, quantitative data and mechanistic insights on the impact of (combinations of) stresses on germination and outgrowth heterogeneity within spore populations are required. In chapter 5 we describe the heterogeneity in outgrowth of a dormant *B. cereus* spore population, sorted to single spore level by using flow cytometry, under (sorbic-) acid-stress and unstressed conditions. From the study described in this chapter, a few observations were made: 1) mild (acid) stress induces a short lag time to initiation of germination, 2) mild stress amplifies germination and outgrowth heterogeneity inherent in the spores, with a positive correlation between increased heterogeneity and the severity of stress factors and 3) mild heat-treatment activated spores, accelerated germination and reduced variance under the stress conditions that induced high levels of heterogeneity in non-heat-treated spores. In addition, this work showed that germination efficiency is not a good predictor for heterogeneity in final outgrowth. This underlined the importance of the first stages of outgrowth under stress conditions, and the Anopore method described in Chapter 6 proved a suitable method to study this. This work provided quantitative information on heterogeneity and showed that within a population of heat-treated spores a

PhD Thesis Clint van Melis_corrected1.indd 115 4-12-2013 10:56:03

large fraction was arrested in the early outgrowth stage, and that outgrowing cells displayed large heterogeneity. Only a small fraction of the population was committed to fast outgrowth under these conditions. In addition, we showed that the initial outgrowth phase is an important factor in the heterogeneous behaviour of a spore population, whereas in other studies the main focus has been on the germination phase (Brousolle et al., 2008; Cronin & Wilkinson, 2008; Stringer et al., 2011; Wang et al., 2011a). Taken together, the studies described in Chapter 5 and 6 show that germination behaviour may not be a good predictor for outgrowth heterogeneity, and should therefore not be used as the sole determinant in predictive modelling. The study where we monitored single spores during early outgrowth (Chapter 6) showed that even when the large majority of a population is delayed in outgrowth, a small subpopulation can still grow out relatively fast. In a sampling plan in a food processing environment such small subpopulations may go undetected, which can result in an underestimation of the number of outgrowing spores, resulting in premature spoilage of products. Another overall conclusion of the work described in Chapters 5 and 6 is that exposure to multiple mild stresses simultaneously, e.g. heat-treated spores incubated at low pH with additional stresses such as sorbic acid, can significantly magnify the heterogeneity that is inherent in a spore population. Therefore, the application of multiple mild preservation methods during food processing may increase heterogeneity and further complicate the prediction of spore outgrowth behaviour. A challenge for the future is to find a delicate balance between minimal process design and product quality and safety. Food-grade antimicrobials with characteristics similar to those of the short-chain carbohydrates investigated in Chapter 4 may contribute to improved designs.

5. Conclusions and future perspectives

The results described in this thesis link phenotypic observations at the population level to responses at transcriptome and at single cell/spore-level for *B. cereus* spore germination under sorbic acid- and other mild preservation-stresses. The work described in this thesis provided more detailed insight in the impact of (organic) acids on germination and outgrowth heterogeneity of *B. cereus* ATCC 14579 spores. The next step would be to translate this knowledge to a more diverse strain set including both laboratory strains and undomesticated strains, such as those isolated from environmental or industrial samples. Extending findings to a larger strain set would cover for diversity-aspects in stress-response, and quantification thereof, and lead to a more generic description of mechanisms involved and may contribute to improved prediction of outgrowth under preservation stresses. An additional challenge for the future is to translate data on outgrowth heterogeneity under mild preservation stress that were generated in culture media, to more complex environments such as present in an actual food product.

In this project, we have shown that high concentrations of HSA can completely block germination of *B. cereus* spores in the population but when applied at lower levels, HSA may increase heterogeneity during germination and outgrowth of dormant spores. Sorbic acid exists in equilibrium between its dissociated and undissociated form, dictated by its dissociation coefficient, pKa, and the pH of the matrix. A small increase in the pH of a product, for example during processing, can significantly reduce the concentration of HSA, which may lead to a shift from complete inhibition of spore germination to highly heterogeneous germination and outgrowth of spores within a food product. To illustrate this, if we assume a pH increase for a product from

PhD Thesis Clint van Melis_corrected1.indd 116 4-12-2013 10:56:03

5 to 5.5, the concentration of HSA drops from 3 mM (blockage of germination) to 1.26mM, which enables *B. cereus* spores to grow out and initiate vegetative growth (unpublished data). In addition, in many food products water and fat-particles will have a non-homogeneous distribution, resulting in microenvironments with relatively high or low concentrations of undissociated acid, and in case of the latter, outgrowth opportunities may arise when concentrations are low enough. It is a challenge to find an acceptable reduction of additives without compromising product stability. An alternative approach could be to aim for induction of germination instead of inhibition. Germinated spores have lower heat resistance properties compared to dormant spores (Setlow, 2003), and it has been postulated repeatedly in the last decade that an activation-germination-inactivation approach can be used for improved control of spores in foods (among others: Akhtar et al., 2009; Brown et al., 1979; Cho et al., 1999; Considine et al., 2008). Løvdal et al. (2011; 2013) describe such an approach for the inactivation of a range of *Bacillus* strains in two different model foods. These authors show that a mild heat-shock at 70^{o}C, followed by short incubation at an intermediate temperature stimulated spores to germinate, enhancing reduction of spore counts at a subsequent heating step at 80-90^{o}C compared to a single heat treatment step of 95^{o}C. An alternative approach that currently also gains attention in literature, is a two-step inactivation approach that uses high hydrostatic pressure to induce germination, followed by inactivation of spores via heat-treatment (Reineke et al., 2013; 2012). In such an approach, media or food products containing spores are preheated to moderate treatment temperatures (>60^{o}C), followed by a high pressure treatment. In the spores, Ca-DPA release is induced by the high pressure, which is followed by water uptake with the concomitant loss of heat resistance, which in turn enables inactivation by the combination of pressure and heat (Paidhungat et al., 2002; Reineke et al., 2011; 2012; Wuytack et al., 1998). Compounds like sorbic acid could add to the safety of products processed with such approaches, but only when the concentration of undissociated acid is aimed at low to intermediate levels, where the germination properties of spores are not affected, but outgrowth is still impaired. This could provide a safety net to tackle those subpopulations of spores that were not 'lured' to germinate during the activation procedures. In order to make such a combined approach feasible, optimal activation conditions and underlying mechanisms still remain to be elucidated, and extensive efforts will be required to design dedicated germination-triggering and inactivation conditions that allow for optimal control of spores in foods.

Many studies on heterogeneity in spore germination and outgrowth have focussed on the effects of a single stress-factor, such as heat, salt or acid (among others: Stringer et al., 2005; Webb et al., 2007; Smelt et al., 2008; Cronin and Wilkinson, 2007; 2008; Zhang et al., 2009; 2010; Wang et al., 2011a; 2012). In a food processing environment, bacterial spores are commonly exposed to more than one stress factor, often applied simultaneously. In order to better understand the impact of stresses that spore-formers experience during preservation, it would be desirable to include combinations of multiple stress factors. The relevance of testing combined stresses is illustrated by the data presented in Chapter 6, showing that combinations of single stresses with a minor impact on heterogeneity resulted in a considerable increase in outgrowth heterogeneity. We specifically noted a dramatic effect of mildly acidic pH (pH 5.5) on outgrowth of heat-treated *B. cereus* ATCC14579 spores not present in heat-treated spores allowed to grow out at pH7. A possible explanation could be that damage repair systems were

PhD Thesis Clint van Melis_corrected1.indd 117 4-12-2013 10:56:03

specifically affected under the mild pH stress conditions. In order to verify this hypothesis, modifications to the Anopore approach, combined with other techniques may provide insights in underlying mechanisms. One approach could include the use of specific (bio)markers to visualise the physiological state of individual cells or germinating spores, e.g. whether a cell/spore experiences stress or whether damage repair mechanisms are activated. The recent development of working fluorescent protein reporter constructs (Eijlander and Kuipers, 2013; Veening et al., 2008), e.g. genes encoding GFP, YFP, or mCherry co-transcribed with selected (bio)marker genes could be used for this purpose. Such reporter-GFP fusions have recently been used successfully in combination with time-lapse fluorescence microscopy in *B. subtilis* and *B. cereus* cells and spores to visualize the heterogeneous expression of selected reporter-genes (de Jong et al., 2010; 2011; 2012; Eijlander and Kuipers, 2013; Veening et al., 2006). A similar approach, but with (bio)markers specific for stress-response events or activation of repair mechanisms, could also be used to study the molecular background of the observed increase in heterogeneity under stressed outgrowth conditions. A limitation of the current time-lapse fluorescence microscopy platforms is that technical constraints prevent the monitoring of long term outgrowth and high throughput of samples. The study recently published by Pandey et al. (2013) showed a sampling approach that, combined with microscope automated fluidic systems (Ducret et al., 2009) may overcome such limitations. As an alternative, Flow cytometry with Fluorescence Activated Cell Sorting (FACS) may provide additional opportunities, since the fluorescent signals emitted by activated GFP-reporter fusions can also be detected by sensors in the FACS. Single cells (or germinating spores) and subpopulations with high or low expression levels of the selected reporter genes can be individually sorted in liquid media and/or on agar plates and studied, for example to study the behaviour of single cells during early outgrowth on Anopore strips, or long term outgrowth in 96/384 wells plates using selected media and/or (model) foods, without or with added preservatives. An additional advantage is that such incubations can be done at a range of relevant temperatures, which allows the application of multiple (stressed) outgrowth conditions. The use of these two approaches, either applied individually or in combination, may provide us with insights that are required to understand (stress-magnified) outgrowth heterogeneity.

At the start of this project, the phenomenon that spore germination and outgrowth may occur as a heterogeneous process was known but far from understood. In addition, knowledge on the impact of (organic) acid stress on germination and outgrowth of *Bacillus* spores was limited. In the past years, work described in this thesis and that by other colleagues has contributed valuable insights in the impact that (sorbic) acid stress can have on germination and outgrowth of spores, and in particular in the mode of action of sorbic acid and impact on heterogeneity during early outgrowth induced by preservation stresses, including heat treatment and sorbic acid. The results obtained in this project may contribute to the rational design of new concepts for improved food preservation and safety.

PhD Thesis Clint van Melis_corrected1.indd 118 4-12-2013 10:56:03

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PhD Thesis Clint van Melis_corrected1.indd 121 4-12-2013 10:56:03

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PhD Thesis Clint van Melis_corrected1.indd 126 4-12-2013 10:56:04

PhD Thesis Clint van Melis_corrected1.indd 127 4-12-2013 10:56:04

PhD Thesis Clint van Melis_corrected1.indd 128 4-12-2013 10:56:04

Samenvatting

Zwak organische zuren, waaronder sorbinezuur, melkzuur en azijnzuur, worden veelvuldig gebruikt in de levensmiddelenindustrie als conserveringsmiddel met als doel de groei van micro-organismen te beheersen. De huidige trend waarbij levensmiddelen minder intensief worden bewerkt creëert kansen voor spore-vormende bederf- en ziekte-gerelateerde micro-organismen, zoals Bacillus cereus. Dormante sporen van *B. cereus* kunnen voedselproductie-condities overleven en vervolgens uitgroeien in een product en op deze manier het risico vergroten op vroegtijdig bederf of de veiligheid van een product aantasten. Het is daarom nodig om additionele conserveringshordes in te zetten, zoals bijvoorbeeld door toevoeging van zwakke organische zuren, om de kwaliteit en veiligheid van een product te kunnen garanderen.

Sorbinezuur wordt vaak als een antimicrobiële component toegevoegd aan producten omdat het zeer effectief is in het remmen van de groei van bacteriën en andere bederf gerelateerde organismen. De hoge effectiviteit wordt mede bepaald door het sterk hydrofobe karakter, dat een additionele inhibitie geeft in vergelijking met andere, minder hydrofobe, organische zuren zoals melkzuur en azijnzuur. De manier waarop sorbinezuur de ontkieming en uitgroei van sporen beïnvloedt werd in dit project bestudeerd op het niveau van genexpressie en dit werd gelinkt aan specifieke fenotypes die optreden tijdens sorbinezuur-geïnduceerde stress afhankelijk van de gebruikte concentratie. De verschillende fasen van spore-ontkieming en -uitgroei werden gekenmerkt door specifieke genexpressie profielen, waaronder sets van genen betrokken bij modificaties aan de buitenste lagen van de cel, transport van nutriënten en aminozuur-metabolisme. Hoge concentraties van sorbinezuur (3mM ongedissocieerd zuur, HSA) blokkeerden volledig de ontkieming via nutriënten-receptoren van *B. cereus* ATCC 14579 sporen. Deze blokkering was reversibel en kon daarnaast omzeild worden door middel van bekende fysische en chemische stimuli, die ontkieming activeren op een nutriënten-receptor onafhankelijke wijze. Dit suggereert dat HSA mogelijk door middel van accumulatie in de binnenmembraan interfereert in de signaaltransductie die plaats vindt tussen nutriënten-receptoren en de zogenaamde SpoVA-kanalen die een rol spelen in de uitlek van componenten tijdens ontkieming van de sporen. Additionele experimenten met andere remmende componenten, waaronder organische zuren en alcoholen met een vergelijkbare structuur, toonden aan dat de lipofiele eigenschappen een belangrijke rol spelen in de ontkieming-blokkerende werking van deze stoffen. Op basis van deze resultaten is aan de hand van de huidige kennis over de interacties die plaats vinden binnen eiwit clusters betrokken in het ontkiemingsproces, een hypothetisch model opgesteld dat de ontkieming-remmende werking beschrijft van sorbinezuur en andere lipofiele componenten.

Sorbinezuur kan, naast ontkieming, ook de heterogeniteit beïnvloeden tijdens uitgroei, mits concentraties worden gebruikt die ontkieming nog toe laten (0.25 – 1.5mM ongedissocieerd zuur). Met name de eerste fasen van de uitgroei bleken heterogeen plaats te vinden wanneer sporen werden bloot gesteld aan meerdere stress-factoren tegelijk.

PhD Thesis Clint van Melis_corrected1.indd 129 4-12-2013 10:56:04

Heterogeniteit in ontkieming en uitgroei was het meest uitgesproken in aanwezigheid van gecombineerde stress condities waarbij hitte-behandelde sporen werden blootgesteld aan een lage pH. Onder deze condities was een grote subpopulatie sporen vertraagd in de stap tussen initiële ontkieming en zwelling en verdere uitgroei.

Voor de levensmiddelenindustrie zijn betrouwbare parameters die het gedrag van overlevende sporen in een levensmiddel kunnen voorspellen gewenst. De data die in dit proefschrift worden beschreven laten zien dat de snelheid van ontkieming geen goede voorspeller is voor heterogeniteit in uitgroei wanneer deze als enige indicator wordt gebruikt. Het werk dat hier beschreven wordt werd verricht met als doel een beter begrip te verkrijgen over het effect van stress condities die relevant zijn tijdens de productie en bewaring van levensmiddelen op de ontkieming en uitgroei van *B. cereus* sporen en de heterogeniteit daarin. De resultaten verkregen uit dit werk hebben een beter inzicht gegeven in de effecten van sorbinezuur en andere lipofiele componenten op *B. cereus* sporen ontkieming en uitgroei.

PhD Thesis Clint van Melis_corrected1.indd 130 4-12-2013 10:56:04

Dankwoord / Acknowledgements

Het zit er op! Het was de afgelopen jaren flink aanpoten, maar het uiteindelijke resultaat is mijn eigen boekje! Zonder de hulp van anderen was mij dit echter nooit gelukt, en daarom wil ik een aantal mensen bedanken voor de energie die ze in mij en/of mijn werk hebben gestoken.

Allereerst wil ik Tjakko, Masja en Roy bedanken voor de kans die ze mij hebben gegeven om dit resultaat te kunnen bereiken. Zonder jullie geduld, ideeën en enthousiasme was dit boekje er nooit gekomen. Ik heb veel van jullie geleerd op professioneel gebied en daar ben ik jullie uitermate dankbaar voor. Onze gezamenlijke inspanningen hebben een aantal mooie artikels opgeleverd. Ik wil hier ook Heidy bedanken voor haar uitvoerige bijdrage aan de laatste twee papers over heterogeniteit.

Greetje, Xiao, Sachin and Lisa. We all started our PhD's in 2008 and we therefore understood each other's obstacles like nobody else could. We shared a lot of fun and not-so-fun moments and we tried to help each other where we could, both work-related or non-work related. Thank you for your company during these years! And the same, of course, also applies to the other colleagues of the Food Microbiology department. Thank you all for your presence during the many coffee breaks, labtrips, dinners, drinks and private conversations. You all made my stay in the department that much more pleasant!

Marcel en Maarten, jullie waren beiden mijn lab-genoten en jullie hebben elkaar afgewisseld als kamergenoot. Ik heb veel van jullie expertise in het lab geleerd en onze talloze gesprekken (vaak onder het genot van een biertje en/of spelletje) hebben mij vele malen een stapje verder geholpen, zowel werk gerelateerd als privé.

Naast de collega's heb ik ook veel steun gehad van een groot aantal andere mensen. Zo wil ik Doda en de andere capoeirista's van onze groep bedanken voor de uitlaatklep die ze mij hebben gegeven; niets helpt beter tegen stress dan jezelf fysiek uitputten door even lekker te vechten (zonder daar iemand mee te verwonden, natuurlijk!). De menige (bord)spelletjes hebben mij geholpen door mijn gedachten even op iets anders te richten dan promoveren. Guys, bedankt daarvoor! And, not to forget, the many dinneraki's that I had with the Greek girls. Your company (and the delicious foods!) have further enriched my life!

Pascal, jij hebt als huisgenoot tijdens de eerste jaren van mijn promotieonderzoek veel mogen genieten van mijn gemopper als ik tegen het zoveelste obstakel aan liep. Ondanks dat je mij vaak niet direct kon helpen, hebben jouw gezelschap en luisterend oor mij toch vaak verder geholpen. Jouw kookkunsten boden met regelmaat uitkomst als ik even naar huis kwam tussen het werk en de capoeiratraining door. Bedankt! Ik ben blij dat jij een van mijn paranimfen wilde zijn.

PhD Thesis Clint van Melis_corrected1.indd 131 4-12-2013 10:56:04

Spence, mijn grotere kleine broertje. Niemand kan tippen aan de duur en sterkte van onze vriendschap. Al van jongs af aan ben jij er voor mij als iets mij dwars zit, en dat is de laatste jaren niet anders geweest. Ik ben daarom trots om jou aan mijn zijde te hebben als paranimf.

Pap en Mam, jullie hebben altijd in mij geloofd en jullie hebben mij geleerd om nooit op te geven, hoe moeilijk het ook is. Ondanks dat jullie weinig begrepen van wat ik allemaal deed hebben jullie toch altijd interesse getoond en een luisterend oor geboden wanneer het mij even teveel dreigde te worden. Door jullie motivatie om te gaan voor wat ik wil, ongeacht de mening die anderen daar over hadden, mag ik mijzelf nu Doctor noemen!

And last, of course, Sonia. Thank you for being my Agapi. Your love and understanding have always helped me to put my problems in perspective. You have kept me going, simply by being there for me and listening to my frustrations. I am happy to have you in my life and I am looking forward to continue our adventures together, wherever they may go!

Clint.

PhD Thesis Clint van Melis_corrected1.indd 132 4-12-2013 10:56:04

List of publications

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Overview of completed training activities

Discipline specific activities

Courses

Systems biology: Statistics of ~omics data analysis (2008)
Advanced visualization, integration and biological interpretation of ~omics data (2009)
Genetics and physiology of food-associated microorganisms (2010)

Other activities

Spore 2009 (oral presentation), a conference on spore-forming food pathogens, Quimper, France (2009)
TI Food & Nutrition Project Meetings and WE-days (2008-2012)
Training periods at other laboratories (a.o. Unilever and TNO, 2009-2011)

General courses

VLAG PhD Week (2008)
Working with Endnote (2008)
Project planning and time-management (2008)
Mobilizing your Scientific network (2011)
Career perspectives (2012)

Other activities

Preparation PhD research proposal (2008)
FHM PhD trip to Canada (2008)
Organization PhD trip to Switzerland (2010 – 2011)
FHM PhD trip to Switzerland (2011)
Food Microbiology department seminars (2008 – 2011)

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Curriculum Vitae

Clint van Melis was born on October 29th, 1978 in Venray, The Netherlands. He received his VWO beta diploma (Dutch, English, Latin, Mathematics, Physics, Chemistry and Biology) at the Raayland College in Venray in 1997. From 1997 to 2003, he studied Biology with a specialization in Microbial Ecology at the Radboud University in Nijmegen. As part of this study, Clint performed an internship at DSM Food specialties in Delft and he wrote his thesis at the Department of Microbiology at the Radboud University. In 2006, Clint started a master study in Biotechnology at the Wageningen University, with a specialization in molecular and cellular biotechnology. His thesis was carried out at the Laboratory of Food Microbiology at Wageningen University and focused on the acid-stress response of different strains of *Bacillus cereus*. After graduating in 2007, he worked for four months as a research assistant at Food & Biobased Research of the Research Centre of Wageningen University. In 2008 he started his PhD project entitled: "Impact of sorbic acid and other mild preservation stresses on germination and outgrowth of *Bacillus cereus* spores". The results of that project are described in this thesis. In June 2013, Clint started working as a junior Post-doc for TI Food & Nutrition, allocated to the Laboratory of Food Microbiology at Wageningen University.

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The study presented in this thesis was performed within the framework of TI Food & Nutrition.

Financial support from Wageningen University and TI Food & Nutrition for printing this thesis is gratefully acknowledged.

Cover design by Clint van Melis.

Printed by Ipskamp Drukkers, B. V., Nijmegen, The Netherlands

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